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STUDIES OF HEPATIC STRUCTURE AND FUNCTION BY FLUORESCENCE MICROSCOPY*

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Received for publication March 17, 1952

An earlier investigation was devoted to the mode of excretion of fluorescein by the frog's liver under normal and abnormal conditions, as observed *in vivo* with the fluorescence microscope (1); and was an outgrowth of unsuccessful attempts to confirm certain aspects of the work of Hirt, Ansorge and Markstahler (2, 3, 4, 5). The normal sequence of events proved to be as follows: After injection (30 mg. per kg.) into the dorsal lymph sac, fluorescein appears promptly in the plasma, then in the hepatic cells and the bile canaliculi. At the peak of excretion, which is attained in approximately 1 hour at c. 15° C., all axial canaliculi and their intercellular branches are sharply delineated at high intensity, and all hepatic cells exhibit diffuse cytoplasmic staining at relatively low intensity. The nuclei are apparently routinely spared by the dye. From the peak of excretion to complete elimination from the liver, the sequence is essentially a progressive diminution in fluorescent intensity to non-detectable levels, achieved first in the hepatic cells, then (much later) in the canaliculi. As long as the canaliculi are delineated they persist as a continuous system, without detectable interruptions in their course. Elimination is usually complete in 24 hours or less. Localized enlargements of the axial canaliculi and dilatations at the distal ends of their intercellular branches are repeatedly observed, and are regarded as normal variants of the canalicular picture during the excretion of fluorescein.

This re-investigation failed to confirm the findings of Hirt *et al.* in the later stages of the excretory process. Following complete delineation of the canalicular system, they described in both frog and rat—and interpreted as normal—an extraordinary "sprouting" of small fluorescein-containing vacuoles from the canaliculi, followed by discontinuity of the canaliculi and widespread intracellular vacuole formation (Figs. 2–7). It was clearly demonstrated that in the

*The "Ultropak" microscope and accessories used for this investigation were made available on loan from the Armed Forces Institute of Pathology, through the courtesy and interest of General Raymond O. Dart, former Director and General Elbert DeCoursey, Director. The expenses of the investigation were defrayed by research funds generously given by Mrs. William G. Baker, Jr. We are indebted to Miss Virginia V. Croker for patient secretarial assistance with the protocols, and to Miss Ellen G. Corddry for assistance in some of the experiments. This paper was presented, in abbreviated form, before the joint meeting of the Society of Clinical Surgery and the Halsted Club in Baltimore on February 6, 1952.

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frog's liver Hirt *et al.* unwittingly concerned themselves in large measure with abnormal phenomena. Prolonged observation of the liver *in vivo* with the fluorescence microscope has proved to be fraught with danger, and distortion of normal function is the rule rather than the exception. Under such conditions the entire range of their abnormal findings was encountered in urethanized frogs, and the clarity of the pictures obtained left no doubt that their illustrations faithfully reproduce what they observed. It was further shown that these aberrant phenomena could develop in pithed frogs, injected with fluorescein and laid aside for hours before exposure and study of the liver. If any single factor plays an essential rôle in their development, anoxia seemed the most likely possibility.

Another aspect of Hirt *et al.*'s work is their insistence upon the occurrence of what may be called a refractory period in the secretory activity of the liver. Specifically, they claim that if—at the end of the excretion of a single dose of fluorescein—the dye is again injected (even intravenously), it will appear in the hepatic cells but will not be excreted into the bile canaliculi until several hours have elapsed. Although their supporting data were apparently all derived from urethanized frogs subjected to prolonged observation, they attribute such a refractory period to the liver of the rat as well. This is indeed a novel concept, and clearly at variance with generally accepted notions concerning normal hepatic function. With precautions to insure experimentally acceptable normal conditions, it has been exhaustively tested in the frog, with consistently negative results (1). As far as the frog's liver is concerned, not only is the concept entirely without basis in fact, but the data adduced in its support afford striking additional evidence of the gross distortion of liver function which may occur in anesthetized animals observed for prolonged periods under the fluorescence microscope.

The present investigation includes further observations upon the amphibian liver, and extends the earlier study to the mechanism of biliary excretion in mammals under normal and abnormal conditions. In both vertebrate forms, three fluorescent compounds of quite different chemical structure—fluorescein, esculin and thioflavine S—have been used for delineation of the bile canaliculi. All of them are rather rapidly transferred in high concentration to the canalicular system, which can be sharply visualized in all its details by the technic of fluorescence microscopy. Limited observations have been made upon the response of the liver to two basic fluorescent dyes, acriflavine and proflavine, as a supplement to the earlier work of Hirt *et al.* upon "trypaflavin".

Singer, who used esculin extensively in studies of the frog's kidney (6, 7, 8), was apparently the first to detect the delineation of the bile canaliculi by this compound (7). Thioflavine S exhibits marked photosensitivity, and this has previously been reported by Singer, who describes striking photodynamic effects upon the renal glomeruli of the frog (6, 9). He makes brief mention of

similar effects in the liver, but states that "it did not seem to affect the color or appearance of the tubuli (bile canaliculi) or their contents" (6). Discussion of other pertinent literature will be deferred until after presentation of the observations.

MATERIAL AND METHODS

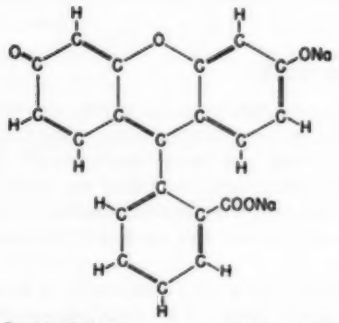
Frogs (*Rana pipiens*), principally male specimens, were obtained at intervals from Vermont, and were maintained (unfed) in a standard ranarium, with running water, until required for study. Adult male salamanders (*Triturus viridescens*) were collected locally. In both amphibian forms, after pithing of the brain and spinal cord, the animal was swathed in water-soaked cotton and the liver was gently exteriorized, without being touched at any time, through a mid-line abdominal incision. The experiments were conducted in a room maintained at c. 21°C.

For the mammalian studies we have used primarily the white mouse and to a lesser extent the white rat (exclusively male specimens in both cases), with single experiments upon the rabbit and monkey. With few exceptions, the mice (strain CF1) and rats were obtained from Carworth Farms, and were maintained on a diet of Purina Laboratory Chow. Our experience with the frog had made us quite aware of the potential dangers of anesthesia, prolonged observation of the liver under the fluorescence microscope, etc. In the frog the simple and rapid procedure of pithing makes it possible to carry out *in vivo* observations upon the liver at any stage of the excretory process. There being no ready means to accomplish this in the mammal, we decided to rely primarily upon initial observations of the liver in animals sacrificed at varying intervals after administration of the fluorescent compound, and brought under the fluorescence microscope as rapidly as possible following exposure of the liver. Mice were sacrificed by crushing of the cervical cord, rats by the same method or by decapitation after being stunned by a blow upon the head. Supplementary *in vivo* observations were made in a relatively small number of mice and rats anesthetized with chloral hydrate (mice—450 mg. per kg., 2% solution; rats—300 mg. per kg., 10% solution; injected intraperitoneally) or nembutal (mice only, injected intravenously in required amount). In such animals the respiratory movements make it extremely difficult to stabilize the liver sufficiently to permit continuing observations at sharp focus, particularly at high magnifications.

The samples of fluorescent compounds used are identified as follows: *Fluorescein sodium*, water soluble (uranine), Coleman and Bell Co., lot nos. 70348 and 480347. *Esculin*, highest purity (hydrate), Pfanstiehl Chemical Co., lot no. 2305. *Thioflavine S*, for biological staining, C.I. No. 816, Hartman-Leddon Co., lot no. 7. *Acriflavine (neutral) N.F. VIII*, a mixture of 2,8 diamino-10-methylacridinium-chloride and 2,8 diaminoacridine, National Aniline Division, Allied Chemical & Dye Corp., lot no. 12711. *Proflavine N.F. IX*, diaminoacridinium-monohydrogen sulfate, National Aniline Division, lot no. 11784. The generally accepted structural formulae for these compounds are given in Figure 1. The formula for bromsulphalein, the excretion of which is widely used as a test of hepatic function in man, is included for comparison.

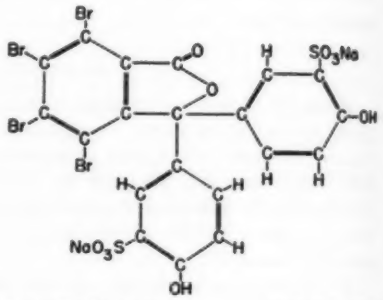
Unless otherwise specified, all solutions of fluorescent compounds were prepared in physiological saline, either Holtfreter's solution (for amphibia) or mammalian Ringer's. Solutions of fluorescein-sodium are stable, but the others deteriorate with time and should be freshly prepared at frequent intervals. Esculin has limited solubility in cold mammalian Ringer's, but dissolves readily on the addition of a minimal amount of alkali (not exceeding 4 drops of N NaOH per 100 ml. of 1:1,000 solution). The routes of administration

FLUORESCEIN - Na SALT (Uranine)



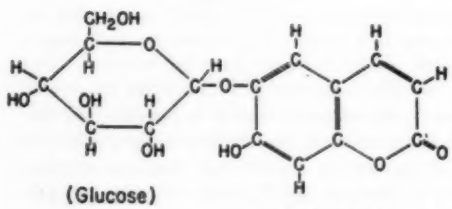
$C_{20}H_{10}O_5Na_2$ Mol. Wt. 376

BROMSULPHALEIN



$C_{20}H_8Br_4O_{10}S_2Na_2$ Mol. Wt. 838

ESCULIN

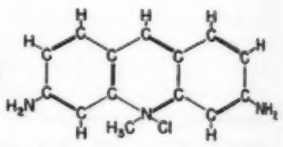


(Glucose) (Esculetin)

$C_{15}H_{16}O_9 \cdot 1\frac{1}{2}H_2O$ Mol. Wt. 340(367)

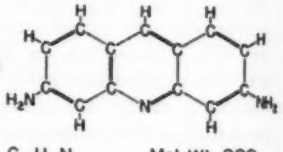
ACRIFLAVINE - A MIXTURE

(I)



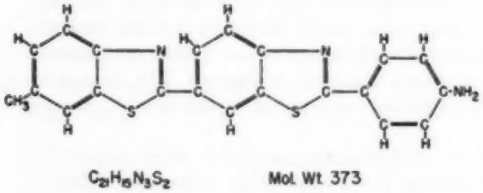
$C_{14}H_{14}N_3Cl$ Mol. Wt. 260

(II)



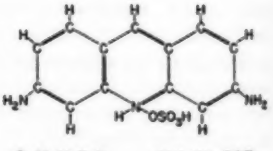
$C_{13}H_{11}N_3$ Mol. Wt. 209

THIOFLAVINE S - A MIXTURE, PRINCIPALLY A METHYLATED AND SULPHONATED DERIVATIVE OF PRIMULIN BASE:



$C_{21}H_{19}N_3S_2$ Mol. Wt. 373

PROFLAVINE



$C_{13}H_{13}N_3O_4S$ Mol. Wt. 307

FIG. 1

were as follows: frog—dorsal lymph sac with few exceptions (intramuscular); salamander—intraperitoneal and intramuscular; mouse—intraperitoneal, intravenous (tail vein) and subcutaneous; rat—intraperitoneal and intravenous (femoral vein, exposed under anesthesia).

The microscope used for this investigation was a Leitz "Ultropak" (research stand UAST 52), designed for work with incident light. The unit was modified by replacement of the usual silvered glass annular mirror by one with an aluminized surface, which proved to be far more efficient. The bulk of the recorded observations were made with the 23×, 55× and 90× water immersion objectives (without immersion caps) and, unless otherwise specified, with the monocular attachment and 6× ocular. Although sharp pictures were frequently obtained with the 10× monocular, the intensity of the fluorescent pictures was usually so much reduced with the binocular attachments as to limit their usefulness. This disadvantage could have been readily overcome by increasing the intensity of the incident light beam, but this was regarded as unwise if deleterious effects upon the liver were to be kept to a minimum. Protective filters (Corning #3-73, polished, standard thickness) were inserted into the oculars to shield the eyes of the observer from reflected ultraviolet light. Quartz annular condensers were not available for the microscope, but the optical glass used by the manufacturer proved to be sufficiently transparent to ultraviolet for present purposes.

Contact between the water immersion objectives and the liver was maintained with either Holtfreter's (amphibia) or mammalian Ringer's solution. To give the observer complete control over this contact at all times, a specially shaped hypodermic needle was securely attached to the microscope, with its tip a short distance from and on a level with the front lens of the objective. The needle was connected by tubing to a syringe, which could be placed to suit the observer's convenience, and from which fluid could be delivered at will.

As a light source we have used the General Electric Co.'s 100-watt, mercury vapor, prefocused spot lamp, formerly designated as C-H4 and now identified by their code as H100-SP4-Projector Spot-100W-PAR 38. The lamp is provided with an aluminum reflector and a clear lens, and supplies a beam of small cross-sectional area and high intensity. A heat-resistant red-purple filter (Corning #5874, polished, standard thickness) was used to remove most of the visible light rays from the beam, which was then passed through a large Pyrex cuvette with flat sides, and filled with boiled distilled water (to absorb heat rays). The light source, filter and cuvette were housed in a lightproof box, and all observations were carried out in a dark room. The emergent, horizontal beam, which was completely shielded from the observer, was aligned at an angle of 45° with the aluminized annular reflecting mirror, which was then rotated laterally in its housing to the position (empirically determined) giving maximum intensity and clarity of the fluorescent pictures. As required by the construction of the microscope, once the light source was properly aligned with the annular mirror, all coarse focusing was done by raising and lowering the microscope stage. Fine adjustment of focus was accomplished in the usual way. On theoretical grounds our light source and optical arrangements are far from ideal, but in practice they have proved to be entirely satisfactory for the present problem.

The lamp used for direct radiation of the liver surface in the experiments with acriflavine was the Hanovia Utility Quartz Mercury Vapor Lamp.

The following conditions apply to the data recorded in Tables: Fluorescein was administered in the form of either a 1:500 (Tables I, II and VI) or a 1:250 (Table III) solution. Esculin (Table IV) and thioflavine S (Table V) were administered in the form of 1:1,000 solutions. All solutions were freshly prepared. All observations recorded in each series were

made in the same experimental period by the same observer, using the 6× monocular and either the 55× or 90× (Table III only) water immersion lens (in each case at the same setting of the ring condenser throughout). After a period for warming up, the light source was operated continuously during each experimental period to minimize variations in intensity. All mice were males of the same strain (CF1), as were the rats (Carworth Farms). Following injection, each animal was maintained in a separate cage on elevated wire screening to minimize the opportunity for ingestion of excreta containing the fluorescent compound. All animals were deprived of food and water following injection except mice nos. 88–91 (Table I) and nos. 164–172 (Table V), which were allowed water *ad lib*. Mice were sacrificed by crushing of the cervical cord, rats by decapitation after being stunned by a blow upon the head. Following free exposure of the liver, with scrupulous avoidance of its surface, the anterior thoracic wall was removed to insure adequate clearance for the large objective, and the heart was incised to avoid transmitted movements. The liver was then supported and somewhat elevated on a saline-moistened flat plate, which was gently introduced under its dorsal aspect and rigidly secured to the side of the metal animal tray. This latter procedure is essential to insure prompt and continuing visualization of the liver at sharp focus, uninterrupted by transmitted peristaltic or other movements, and more than justifies the additional time and effort required. Finally, the exposed surface of the liver was gently washed with Ringer's solution, to remove any surface residual of the compound injected intraperitoneally. The delay between time of sacrifice and initial visualization of the liver under the microscope varied, for Table I, between 1 min. 45 sec. and 2 min. 20 sec.; for Table II, between 1 min. 50 sec. and 3 min.; for Table III, between 1 min. 50 sec. and 2 min. 25 sec.; for Table IV, between 1 min. 50 sec. and 2 min. 40 sec. except in mouse no. 138 (3 min. 10 sec.); for Table V, between 1 min. 52 sec. and 2 min. 35 sec.; for Table VI, between 1 min. 30 sec. and 2 min. 5 sec. In all animals observations were confined to the superficial hepatic cells and bile canaliculi on the ventral surface of the same (left lower) lobe, and were limited to the first two minutes following initial visualization. When little or no trace of the injected compound could be detected in this lobe, the ventral surface of a second lobe (left upper) was surveyed after the first two minutes, in each instance with essentially the same findings except where otherwise noted in Tables IV and V. The subjective judgments expressed in the fractional estimates in all tables are rough approximations at best, and are to be so interpreted. At the end of observations upon the liver, all animals were carefully examined under ultraviolet light to detect any possible miscarriage of the intraperitoneal injection (none was found), and to evaluate the fluorescent intensity of the injected compound in the gall bladder and the extrahepatic bile passages. The natural fluorescence of the latter (in mice, a soft yellow in color; of low intensity at best in the gall bladder; of low or very low intensity, or absent, in the bile passages) was taken into account in these evaluations.

In special studies carried out upon white mice, it was demonstrated that the various lobes of the liver are approximately equivalent for study of the biliary excretion of the fluorescent compounds employed. Groups of animals received intraperitoneal injections of fluorescein, esculin and thioflavine S respectively, in uniform dosage of 30 mg. per kg. All animals were sacrificed 15 minutes after injection, and were prepared so that the ventral surfaces of three lobes could be rapidly surveyed in turn. The delay between time of sacrifice and initial visualization of the liver varied between 2 min. 7 sec. and 3 min. 10 sec., and the survey of all three lobes was completed within three minutes. All observations were made by the same observer, using the 55× lens and the 6× monocular. In the mammal, as in the frog (1), the conclusion appears justified that observations made upon the ventral surface of either or both of the two lobes (left lower and upper) most accessible for study are acceptable as evidence of the course of biliary excretion in the liver as a whole.

OBSERVATIONS UPON THE AMPHIBIAN LIVER

FROG (*Rana pipiens*)

Natural fluoresence of the liver

The natural fluorescence of the frog's liver has previously been described in detail elsewhere (1, 2, 4, 10), and requires only brief comment. The most striking features of the liver are the occurrence of small fluorescent bodies (yellow or whitish-yellow in color) in the cells lining the hepatic sinusoids, and the fact that their natural fluorescence is rapidly extinguished by exposure to ultraviolet light. With their initial brilliance these bodies may so dominate the microscopic picture as to prevent visualization of small amounts of injected fluorescent compounds in plasma, hepatic cells and even bile canaliculi. Suppression of their fluorescence is so prompt, however, that they have never presented a real problem. The cytoplasm of the hepatic cells exhibits a bluish fluorescence of low intensity. It does not interfere appreciably with recognition of the yellow-green fluorescence of fluorescein, but may do so with detection of small amounts of the characteristic blue fluorescence of both esculin and thioflavine S. The nuclei of the hepatic cells can usually be visualized as dark, relatively non-fluorescent bodies. In our frogs, under our conditions, there is no suggestion of the bile canaliculi.

Studies with fluorescein

Our more recent ***in vivo*** studies of the frog's liver have fully confirmed our earlier observations (1) as far as the normal mechanism of fluorescein excretion is concerned, and need not be described in detail. As a supplement to the data previously reported, the following protocol illustrates the sequence of events which may be observed in a pithed frog, when the liver is held under continuous observation following injection of fluorescein (60 mg. per kg.):

No. 41, female, 31.0 gm., July:

0 Pithed, liver exposed.

3′25″ Ventral surface of left upper lobe widely surveyed in preliminary study of natural fluorescence, first with 23×, then with 90× lens. Picture presents no unusual features. Survey completed at *22′0″*.

26′0″ Injected 0.93 ml. of fluorescein 1:500 intramuscularly, left thigh.

27′30″ Continuous survey of ventral surface of left upper lobe begun, 23× lens. Area of observation shifted frequently throughout.

32′30″ Fluorescein fluorescence first detected in plasma of active vessels, at very low intensity.

38′30″ First faint pictures of bile canalicular delineation encountered. Dye still at very low intensity in plasma, not detectable in hepatic cells. Circulatory activity very good.

43′0″ Delineation of canaliculi has developed steadily in interim. Axial canaliculi delineated almost everywhere, usually at low, sometimes at very low, rarely at moderate intensity. Very few intercellular branches delineated. Dye in plasma still at very low

intensity in sinusoids, at very low or low intensity in larger vessels. Not detectable in hepatic cells.

48′0″ Picture has developed steadily in interim. Canaliculi now delineated predominantly at moderate intensity, intercellular branches more numerous. No change in plasma or hepatic cells.

49′15″ Presence of fluorescein in hepatic cells, at very low intensity, first detected with assurance.

52′30″ Picture has continued to develop. Some canaliculi now delineated at high, remainder still at moderate intensity. Many more intercellular branches delineated. Dye present in many hepatic cells at low, in remainder still at very low intensity.

61′15″ Delineation of axial canaliculi and their intercellular branches now essentially complete. Large majority delineated at high, remainder at moderate intensity. Cytoplasm of hepatic cells uniformly shows fluorescein at low intensity, nuclei can not be visualized at this magnification. No change in plasma. Circulatory activity maintained at high level. Appearance of fluorescein in cytoplasm of hepatic cells is a gradual process, with no perceptible wave of spread of the dye from sinusoidal margin toward canaliculi, and no detectable concentration at sinusoid-hepatic cell interface.

64′0″ No appreciable further development of picture in interim.

65′0″ Survey of ventral surface of left upper lobe continued, 90× lens. Above observations confirmed. In addition, nuclei of hepatic cells can be visualized in most laminae as non-fluorescent areas. Canalicular system displayed with great clarity in all its details. Only occasional small or medium-sized ampulla-like enlargement of axial canaliculi, and small spherical dilatation at distal ends of intercellular branches. No sprouting of fluorescein-containing vacuoles from canaliculi, and no intracellular vacuole formation.

72′0″ Observations terminated.

Studies with esculin

Following injection in adequate dosage, esculin appears promptly in the plasma, to which it imparts a blue or, at higher intensity, whitish-blue fluorescence. Erythrocytes are neatly displayed as dark, non-fluorescent objects. The compound is rapidly transferred in high concentration to the bile canaliculi, which are sharply delineated by the same blue or whitish-blue fluorescence. Against this rich blue background the hepatic cells appear essentially devoid of fluorescence, and in areas of active circulation exhibit an ill-defined brownish cast instead of the bluish color characteristic of their natural fluorescence. Neither nuclei nor other details of structure can be visualized under these conditions, and intercellular boundaries are not apparent. Occasionally, in areas of inactive circulation, the cells may exhibit an at best suggestive esculin fluorescence of the cytoplasm but not of the nuclei, which appear as dark, non-fluorescent areas.

The following observations were made in the month of July upon male frogs (23–28 gm.) pithed at varying intervals following the injection (dorsal lymph sac) of esculin 1:1,000 at uniform dosage of 30 mg. per kg. The delay between pithing and visualization of the liver varied between 2 and 4 minutes.

Observations were confined to the ventral surface of a single (usually left upper) lobe, which was surveyed briefly with the 23×, then at greater length with the 90× lens. The total period of observation varied between 7 and 24 minutes. Emphasis was placed upon speed and accurate evaluation of the liver at the beginning of the period, and it is these initial observations (unless otherwise noted) which are recorded below.

No. 29, 5 min.: Axial canaliculi completely delineated, majority at moderate, few at low, rarely at high intensity. Delineation of intercellular branches variably incomplete. Plasma fluorescence low to moderate in sinusoids, moderate to high intensity in larger vessels.

No. 24, 15 min.: Axial canaliculi and their intercellular branches completely delineated at moderate or high intensity. Plasma fluorescence of moderate to high intensity.

No. 31, 20 min.: Axial canaliculi completely delineated, moderate to high intensity. Delineation of intercellular branches at times variably incomplete. A few localized enlargements of axial canaliculi, variable in size, occasionally quite large.

No. 32, 30 min.: Axial canaliculi completely delineated, usually at moderate or high, occasionally at low intensity. Delineation of intercellular branches variably incomplete from lamina to lamina, and even within same lamina. Occasional localized enlargements of axial canaliculi, variable in size, usually small. A few small dilatations at distal ends of intercellular branches. In areas of inactive circulation, appearance of hepatic cells suggests presence of esculin fluorescence in cytoplasm at a just detectable level.

No. 25, 63 min.: Axial canaliculi completely delineated at moderate or high intensity. Delineation of intercellular branches markedly incomplete, many axial canaliculi essentially devoid of them. A number of localized enlargements of axial canaliculi, variable in size, some quite large. Occasional dilatations, variable in size, at distal ends of intercellular branches. In latter part of observation period noted a number of vacuoles, of small and medium size, sharply delineated by esculin fluorescence, adjacent to axial canaliculi, and at times connected thereto by delicate fluorescent channels.

The following protocol illustrates the sequence of events which may be observed in a pithed frog, when the liver is held under continuous observation following injection of esculin (60 mg. per kg.):

No. 42, male, 26.5 gm., July:

0 Pithed, liver exposed.

3′35″ Ventral surface of left upper lobe widely surveyed in preliminary study of natural fluorescence, first with 23×, then with 90× lens. Picture presents no unusual features. Survey completed at *15′55″*.

18′45″ Injected 1.59 ml. of esculin 1:1,000 intramuscularly, left thigh.

19′20″ Continuous survey of ventral surface of left upper lobe begun, 23× lens. Area of observation shifted throughout.

20′20″ Blue esculin fluorescence first detected in plasma of active vessels at very low, rapidly developing to low intensity.

26′15″ Detected first delicate pictures of bile canalicular delineation. Plasma fluorescence now at moderate intensity. Circulatory activity excellent. Hepatic cells appear dark, with frequently an ill-defined brownish cast.

30′0″ Axial canaliculi now delineated practically everywhere, at from very low to low intensity. Numerous intercellular branches delineated, but many axial canaliculi devoid of them. No change in plasma, hepatic cells or circulatory activity.

37′55″ Picture has developed steadily in interim. Canaliculi delineated at from low to moderate intensity, with many more intercellular branches. No change in plasma, hepatic cells or circulatory activity.

45′45″ Delineation of axial canaliculi and their intercellular branches essentially complete, most at moderate, few still at low intensity. Plasma fluorescence at between moderate and high intensity. No change in hepatic cells or circulatory activity.

48′45″ Canaliculi in many instances delineated at high intensity, and richly provided with intercellular branches. Plasma fluorescence at high intensity. No change in hepatic cells or circulatory activity. Survey with 23× lens completed at *51′45″*.

52′45″ Survey of ventral surface of left upper lobe continued, 90× lens. Above observations confirmed. Esculin not detectable in hepatic cells, nuclei of which can not be visualized. Occasional localized enlargements of axial canaliculi, of small or medium size. No sprouting of esculin-containing vacuoles from bile canaliculi, and no intracellular vacuole formation.

59′45″ Observations terminated.

Esculin has proved to be admirably suited for studies of the mechanism of biliary excretion by fluorescence microscopy, and exhibits none of the marked photosensitivity which characterizes thioflavine S (see below). We have seen no more striking pictures of the frog's liver than those obtained by combining esculin with fluorescein. In a frog injected with both compounds, each at c. 30 mg. per kg., and pithed 30 minutes later, the plasma exhibits the blue fluorescence of esculin; the non-fluorescent erythrocytes are well shown; the bile canalicular system is boldly delineated in a soft, golden-yellow color of high fluorescent intensity; and the whole picture is accentuated by the dull, relatively non-fluorescent, ill-defined yellowish-brown color of the hepatic cells.

Studies with thioflavine S

Following injection in adequate dosage, thioflavine S appears promptly in the plasma, to which it usually imparts an ill-defined yellow or, at higher intensity, whitish-yellow fluorescence; in one instance (frog no. 43, see below) the plasma fluorescence was blue in color. The erythrocytes remain non-fluorescent. The dye is rapidly transferred in high concentration to the bile canaliculi, which are sharply delineated in a color which varies with the intensity of the fluorescence: blue at low, whitish-blue at moderate and bluish-white at high intensity. When present at detectable levels in the hepatic cells, the dye imparts to the cytoplasm a diffuse, homogeneous fluorescence, the color of which varies with intensity as in the bile canaliculi. Even at very low intensity, the blue fluorescence attributable to thioflavine S can be readily distinguished from the bluish natural fluorescence of the hepatic cells. The nuclei are regularly spared by the dye, and by virtue of their non-fluorescence stand out in sharp contrast to the cytoplasm. One frequently observes a delicate line of yellow fluorescence along the margins of the hepatic laminae, suggesting a concentration of the dye at the sinusoid-hepatic cell interface.

Thioflavine S is remarkably photosensitive, and the initially sharp pictures

of the bile canaliculi rapidly decrease in fluorescent intensity and clarity when any microscopic field is held under continuous observation, and disappear within a few minutes. Their disappearance is frequently accompanied by an apparent increase in the fluorescent intensity of the dye in the cytoplasm of the associated hepatic cells. No particular study has been made of photodynamic effects upon the vascular system.

The following observations were made in the month of August upon male (no. 45) and female (nos. 44 and 46) frogs (26–32.5 gm.) pithed at varying intervals following the injection (dorsal lymph sac) of thioflavine S 1:250 or 1:500 at uniform dosage of 60 mg. per kg. The delay between pithing and visualization of the liver varied between 2 min. 30 sec. and 2 min. 50 sec. Observations were confined to the ventral surface of a single lobe, and were made with the 90× lens. The total period of observation varied between 6 and 9 minutes.

No. 45, 10 min.: Bile canalicular system completely delineated, predominantly at high intensity. Cytoplasm of hepatic cells exhibits diffuse blue induced fluorescence of low intensity. Nuclei strikingly displayed as non-fluorescent areas. Plasma in active vessels shows the dye, usually at low intensity, ill-defined yellowish or yellowish-white in color. Erythrocytes clearly outlined. In many instances there appears to be a concentration of the dye, yellowish in color, from low to moderate intensity, at sinusoid-hepatic cell interface. In some instances active sinusoids slowed down and stopped completely in course of relatively brief period of observation.

No. 44, 20 min.: Canalicular system completely but delicately delineated, on the whole at rather low intensity. Relatively little contrast between canaliculi and induced fluorescence of hepatic cells. Outstanding feature of preparation is striking display of hepatic cell nuclei as non-fluorescent bodies. Plasma of all active vessels shows ill-defined yellowish fluorescence of low intensity. In many instances can observe delicate yellowish line, between low and moderate intensity, at sinusoid-hepatic cell interface. Scattered over surface a small number of hepatic cells with intensely fluorescent cytoplasm, bluish-white in color.

No. 46, 30 min.: Axial canaliculi completely delineated, as are most intercellular branches, majority at moderate, few at low, occasionally at high intensity. Cytoplasm of hepatic cells exhibits induced fluorescence of low intensity; nuclei strikingly demonstrated as non-fluorescent areas. Dye detectable in plasma of active vessels, at very low or low intensity, ill-defined yellowish or whitish-yellow in color. In some laminae a thin, yellowish line at peripheral margin, of low intensity at best, suggesting concentration of dye at sinusoid-hepatic cell interface.

The following protocol illustrates the sequence of events which may be observed in a pithed frog, when the liver is held under continuous observation following injection of thioflavine S (60 mg. per kg.):

No. 43, female, 30 gm., July:

0 Pithed, liver exposed.

8′30″ Injected 2.28 ml. of thioflavine S 1:1,000 into dorsal lymph sac.

9′0″ Continuous survey of ventral surface of left upper lobe begun, 23× lens. Area of observation shifted frequently throughout unless otherwise noted. Initially, usual picture of natural fluorescence.

11′15″ Presence of dye first detected in plasma, soft blue in color, at very low, rapidly increasing through low to moderate intensity.

12′55″ Presence of dye first detected in cytoplasm of hepatic cells, having appeared almost imperceptibly in laminae adjacent to active vessels. Circulatory activity initially rather sluggish, gradually improved.

16′25″ Majority of hepatic laminae now exhibit blue induced fluorescence of low, moderate or occasionally high intensity.

17′45″ First suggestive pictures noted of bile canalicular delineation. Only axial canaliculi can be visualized, first at very low, developing rapidly to low intensity.

20′45″ Several areas show widespread delineation of canaliculi, at from very low to moderate intensity. Picture developing rapidly, and particularly striking in areas where hepatic cells exhibit little or no induced fluorescence. In laminae of moderate or higher intensity, often difficult and at times impossible to visualize canaliculi. Up to now no concentration of dye along margins of blood vessels or at sinusoid-hepatic cell interface. Picture suggests gradual appearance of dye at detectable levels throughout cytoplasm of hepatic cells.

29′0″ Hepatic laminae, with rare exceptions, exhibit induced fluorescence at highly variable intensity (from very low to high). Nuclei of hepatic cells completely spared by the dye. Dye still detectable in plasma at low intensity. Canalicular picture now suppressed in large area held under continuous survey; only occasional satisfactory pictures, irrespective of intensity of induced fluorescence in hepatic cells.

34′0″ Observations deliberately shifted to more peripheral portions of lobe, hitherto not observed. Striking pictures obtained, with complete delineation of canalicular system at from low to high (usually moderate) intensity in large majority of laminae. Intensity of induced fluorescence in associated cells uniform in a given lamina, but highly variable in different laminae. Quite apparent that canalicular picture is rapidly destroyed as a given field is held under observation.

39′55″ A hitherto unobserved field, showing apparently complete delineation of canaliculi and little or no induced fluorescence of hepatic cells, held under continuous observation. Fluorescent intensity and clarity of canaliculi decreases progressively, with no detectable change in appearance of associated cells. By *43′20″* only an occasional remnant, and by *44′10″* no trace of canalicular delineation persists.

45′20″ Another field entirely similar to above held under continuous observation. Same progressive decrease in clarity and intensity, and by *50′45″* no trace of canalicular delineation persists. In contrast to previous field, intensity of induced fluorescence in some laminae very definitely increases as canaliculi disappear.

52′55″ Observations terminated.

In summary, in so far as the normal mechanism of biliary excretion and the architecture of the bile canalicular system are concerned, the pictures obtained with esculin and thioflavine S are fundamentally the same in all essentials—exclusive of time and dosage factors, which have not been thoroughly evaluated—with those obtained with fluorescein.

Studies with acriflavine and proflavine

Our observations upon the frog's liver following the injection of acriflavine and proflavine have not contributed to our understanding of the mechanism of biliary excretion. Under the circumstances, although such studies afford

information of considerable importance and interest, they will be reported here in only brief and summary form.

One can achieve with acriflavine, by virtue of its distinctive fluorescence in the cytoplasm, a sharp delineation of individual hepatic cell territories, the cells being separated by dark, non-fluorescent stripes of variable width. With our equipment and our sample of acriflavine, essentially the entire sequence of events—as reported by Hirt *et al.* (2) for trypaflavin—can be induced, though far more delicately than they observed it, by continuous and prolonged observation of the same microscopic field. Their most striking pictures of segregation of the dye in the nuclear membrane and in intranuclear bodies can be obtained at high intensity and with great clarity when the frog's liver is directly exposed to rather intense ultraviolet radiation under a quartz mercury vapor lamp. From our studies, the conclusion is almost inescapable that Hirt *et al.*'s incident light beam, in terms of its composition and intensity, was far more damaging to the liver than ours. We have yet to observe even a suggestive picture of bile canalicular delineation by acriflavine.

Cell territories may be similarly demarcated in proflavine preparations, but again no trace of bile canalicular delineation has ever been observed. With varying dosage and after varying time intervals, the presence of proflavine in the hepatic cell nuclei has been readily apparent and widespread in some animals at the very beginning of observations. The nuclear fluorescence is almost invariably homogeneous, but in one instance with high dosage (120 mg. per kg., pithed 30 minutes later) the initial picture included many nuclei exhibiting segregation of the dye in the nuclear membrane and in intranuclear bodies. We have observed that penetration of proflavine into the nuclei of the hepatic cells may be accelerated by continuous observation of the liver under the fluorescence microscope. With increase in nuclear fluorescence, the delineation of cell outlines is lost and the intensity of cytoplasmic fluorescence is significantly reduced.

SALAMANDER (*Triturus viridescens*)

Supplementary studies of biliary excretion in amphibia have been carried out in the adult salamander, using both fluorescein and esculin. The salamander liver offers particular difficulties for such *in vivo* studies, since visualization of the hepatic cells and bile canaliculi is obstructed, to a variable extent, by the lymphogranulopoietic layer which lies immediately under the liver capsule (11, 12). Except for this cellular layer, the natural fluorescence of the liver is essentially equivalent to that in the frog, and does not appreciably interfere with recognition of either of the fluorescent compounds used. The observations with both fluorescein and esculin indicate that the mechanism of biliary excretion is fundamentally the same in the salamander as in the frog. They have further shown that the architecture of the bile canalicular system is similar in

all important respects to that in the frog, although the course and pattern of the axial canaliculi and their intercellular branches is at times considerably more diverse and less regular.

It has previously been demonstrated that frogs can and do absorb fluorescein from an aqueous environment (1). In a frog exposed for c. 48 hours to a 1:10,000 concentration of the dye, the amount so absorbed may be sufficient to achieve complete delineation of the bile canaliculi at from low to moderate intensity. Even after 6 hours' exposure to a 1:140,000 concentration, all canaliculi may be delineated at low intensity. A number of attempts to achieve similar results in the salamander were almost entirely unsuccessful. Only one of three specimens exposed for whom 18 to 46 hours to a 1:5,000 concentration (the maximum which was tried) showed even partial delineation of the canaliculi, and then in only occasional laminae, at low or very low intensity.

Sturm (13, 14) has reported the delineation, following injection of fluorescein, of the axial bile canaliculi and their intercellular branches in salamander larvae (*Salamandra maculosa*). His description is brief, but entirely convincing; and Ellinger's (15) criticism of this aspect of Sturm's work would appear to be quite unjustified.

OBSERVATIONS UPON THE MAMMALIAN LIVER

Natural fluorescence of the liver

The natural fluorescence of the mammalian liver has been described by others (2, 5, 16), and has been re-investigated as a necessary preliminary to our studies with fluorescent compounds. The pictures obtained in the white mouse and rat are entirely similar to those in the frog (see above) except that, with our equipment at least, the nuclei of the hepatic cells can not be visualized.

Studies with fluorescein

The normal sequence of events in the mammalian liver, following the intraperitoneal injection of fluorescein in dosage of 30 mg. per kg., is illustrated by the data summarized in Tables I (white mice) and II (rats). The sequence with dosage of 60 mg. per kg. is illustrated by Table III (white mice). As in the frog, at the peak of excretion all bile canaliculi are sharply delineated at rather high fluorescent intensity, which exhibits a progressive diminution to non-detectable levels as the dye is eliminated from the liver. In all of the animals recorded in Tables I–III (as well as in Table IV) the canaliculi, wherever delineated, appeared as a continuous system, without interruptions in their course. Furthermore, no instance was encountered of "sprouting" of vacuoles from canaliculi or of intracellular vacuole formation, described by Hirt *et al.* as characteristic of the later stages of the normal excretory process.

TABLE I

Observations upon White Mice Sacrificed at Intervals following Intraperitoneal Injection of Fluorescein 30 mg. per kg.[1]

NO.	WEIGHT	INTERVAL	INTENSITY OF FLUORESCEIN IN BILE CANALICULI			
			High	Moderate	Low	Very low
	gm.	min.				
77	27.1	5	—	Rare	¾	¼
78[3]	35.0	10	Most[3]	Few	—	—
79	35.7	15	Few	Most	Few	—
80	30.6	20	Few	Most	Few	—
81	27.5	25	—	⅔	⅓	—
82	22.1	30	Few	Most	Few	—
83	29.6	45	—	Few	Most	Few
84	24.9	60	—	Few	Most	Few
85	30.3	75	—	—	Rare	⅔[4]
86[5]	27.9	90	—	—	—	—

[1] At end of observations upon liver, fluorescein noted to be present in extrahepatic bile passages and gall bladder at moderate (no. 77) or high intensity (nos. 78–91).

[2] Fluorescein detectable in cytoplasm of hepatic cells, at very low or low intensity (about equal proportions).

[3] Several suggestive instances of delicate "beading" noted in most superficial canaliculi.

[4] Fluorescein not detectable in canaliculi in approximately ⅓ of the area surveyed.

[5] In mice nos. 87–91, 25.3–32.5 gm., studied at 2, 2½, 3, 3½ and 4 hrs. after injection, no trace of fluorescein could be detected in the liver.

TABLE II

Observations upon White Rats Sacrificed at Intervals following Intraperitoneal Injection of Fluorescein 30 mg. per kg.[1]

NO.	WEIGHT	INTERVAL	INTENSITY OF FLUORESCEIN IN BILE CANALICULI			
			High	Moderate	Low	Very low
	gm.	min.				
19[2]	227	10	Few	⅓	⅔	Few
20[2]	226	20	½	½	—	—
21[2]	247	30	½[3]	½	Few	—
22	251	45	⅙	⅔	⅙	—
23[4]	271	60	—	⅙	⅔	⅙
24	222	75	—	¼	½	¼
25[5]	209	90	—	—	—	—

[1] At end of observations upon liver, fluorescein noted to be present in extrahepatic bile passages at moderate (nos. 19, 20, 25 and 26) or high intensity (nos. 21–24).

[2] Fluorescein detectable in cytoplasm of hepatic cells: In no. 19 at moderate (occasional), low (½) or very low (½) intensity; dye not detectable in remaining ½ of cells. In no. 20 at low or very low intensity (about equal proportions). In no. 21 occasionally at low, usually at very low intensity; not detectable in occasional cells.

[3] Occasional suggestive instances of delicate "beading" noted in most superficial canaliculi.

[4] One superficial hepatic cell noted containing fluorescein at high intensity in cytoplasm.

[5] Rat no. 26, 209 gm., sacrificed 2 hrs. after injection, likewise showed no detectable trace of fluorescein in the liver.

The following observations were made upon a white mouse (23 gm.) sacrificed 2 minutes after the intravenous injection of fluorescein 1:100 at a dosage

TABLE III

Observations upon White Mice Sacrificed at Intervals following Intraperitoneal Injection of Fluorescein 60 mg. per kg.[1]

NO.	WEIGHT	INTERVAL	INTENSITY OF FLUORESCEIN					
			In Bile Canaliculi				In Hepatic Cells	
			High	Moderate	Low	Very low	Low	Very low
Series A								
	gm.	min.						
107	19.9	5	Few	1/5	1/2	1/6	1/2[2]	1/2
108	18.8	10	2/5	1/3	Few	—	3/4[2]	1/4
109	20.8	15	2/5	1/3	Few	—	2/5[2]	1/3
110	19.5	20	3/4	1/4	—	—	Most[3]	Few
111	20.0	25	2/5	1/3	Few	—	Most	Few
112	18.5	30	Few	Most	Few	—	Most	Few
113	20.5	40	Few	2/5	1/3	—	Few	Most
114	19.5	50	Few	2/5[4]	1/3	Few	Few	Most
115	21.1	60	—	2/5	1/3	Few	Few	Most
Series B								
116	21.6	75	—	1/6	2/5	1/6[5]	—	—
117	21.3	90	—	Rare	1/4	3/4[5]	—	—
118	20.1	105	—	—	—	1/2	—	—
119	21.2	120	—	—	—	Few	—	—
Series C								
120	21.1	140	—	—	—	Few[6]	—	—
121	20.3	160	—	—	—	Few	—	—
122	22.9	180	—	—	—	Rare[7]	—	—
123[8]	16.1	210	—	—	—	—	—	—

[1] At end of observations upon liver, fluorescein noted to be present in extrahepatic bile passages and gall bladder at low (no. 107), moderate (no. 108) or high intensity (nos. 109–124).

[2] Cytoplasmic fluorescence of moderate intensity in occasional laminae.

[3] General intensity of cytoplasmic fluorescence adjudged to be appreciably higher in this animal than in any of the others.

[4] Several suggestive instances of delicate "beading" noted in most superficial canaliculi.

[5] Bile canaliculi could not be visualized in occasional laminae.

[6] Delineated with extreme delicacy.

[7] Suggestive at best.

[8] Mouse no. 124, 17.2 gm., sacrificed 4 hrs. after injection, likewise showed no detectable trace of fluorescein in liver.

of c. 85 mg. per kg. They are given in some detail to illustrate the pictures of the bile canalicular system which may be obtained with this dye. The delay

between sacrifice and visualization of the liver was 3 minutes, and all observations were made upon the ventral surface with the 90× lens.

No. 24: All bile canaliculi sharply delineated at high intensity. At surface of liver displayed as a more or less continuous polygonal meshwork in plane of focus. Just below surface (first level) give picture suggesting that in frog, with an "axial" canaliculus (showing a zig-zag course in both vertical and horizontal planes) and many lateral "branches" which appear to arise and course between each pair of adjacent hepatic cells. Still further below surface (second level) this picture is frequently replaced by a polygonal pattern in plane of focus, not as sharply defined but essentially as regular as meshwork on surface. Practically all of the "branches" at first sub-surface level can be shown to connect with other canaliculi either in surface polygonal meshwork or in laminae lying still more deeply in substance of liver. Fluorescein not detectable in hepatic cells.

Subcapsular laminae

At the very beginning of our studies of biliary excretion in mammals, we encountered on the surface of the liver pictures which we had not anticipated, but which have proved to be highly characteristic. In the frog's liver, at the most superficial focus, one typically observes a simple and fairly regular alternation of hepatic sinusoids and laminae. When the bile canaliculi are delineated by fluorescein, etc., they can be visualized under the fluorescence microscope with the greatest clarity, in all their details, as can many aspects of the hepatic cells and sinusoids. There is never any indication that the observer's view is being obstructed by any capsular or subcapsular structures interposed between him and the most superficial laminae and sinusoids.

In the mammal, the situation is in large part quite different. The first picture of bile canalicular delineation to come into focus is predominantly one of a more or less extensive, essentially rectilinear, polygonal meshwork. This pattern is displayed in the plane of focus over large areas, and is interrupted in varying degree (at times to a minimal extent) by sinusoidal channels which achieve an immediately subcapsular position. It is obvious, from many of the fluorescent pictures obtained under both normal and abnormal conditions, that each of the polygons in the superficial meshwork delimits a single hepatic cell territory. It is equally obvious that in the mammal, in contrast to the frog, there occurs with great frequency a more or less continuous, superficial or subcapsular sheet or lamina of hepatic cells. By this lamina, which over the underlying sinusoids appears to be only one hepatic cell in thickness, the majority of the sinusoids in a particular area may be denied access to the surface of the liver. Where this is so, the underlying laminae join the subcapsular lamina at approximately right angles. The situation is analogous to that described by Remak (17) and Elias (18) in the "limiting laminae" at the periphery of the hepatic lobules, surrounding the portal spaces.

Having once become aware of the subcapsular laminae in our fluorescence

studies, we found it a relatively simple matter to confirm their existence in routine histological preparations. To the best of our knowledge, the structural relationships involved have not previously been fully appreciated or described. On the other hand, Franke and Sylla (10), many years ago, published a photograph of bile canalicular delineation by fluorescein in the rat's liver (their Fig. 15) which can only be explained in terms of such a subcapsular lamina. Their photograph, if one ignores the abnormal phenomena portrayed, clearly illustrates the superficial polygonal meshwork of bile canaliculi which we have regularly encountered in all mammals examined.

Our reason for emphasizing the occurrence and characteristics of the subcapsular lamina is that the pictures of bile canalicular delineation observed on the surface of the mammalian liver are incomprehensible unless its existence is taken into account. In recording our observations, we routinely describe the appearance of the canaliculi in the polygonal pattern on the surface of the liver and in the underlying laminae. The latter are identified as being at the first, second, third, etc. sub-surface level with progressively deeper focus.

Studies with esculin

The normal sequence of events in the mammalian liver, following the intraperitoneal injection of esculin in a dosage of 30 mg. per kg., is illustrated by the data summarized in Table IV. As with fluorescein, the bile canaliculi always appeared as a continuous system, and no instance was encountered of "sprouting" or of intracellular vacuole formation. The following more detailed observations were made upon white mice (31.5 and 37 gm.) sacrificed 5 and 15 minutes after intraperitoneal injection of esculin 1:1,000 at a dosage of 30 mg. per kg. The delay between sacrifice and visualization of the liver was 3 and 4 minutes, and the length of the observation period was 13 and 16 minutes respectively. The observations were made upon the ventral surface of the left lower lobe, surveyed in turn with the 23× and 90× lenses.

No. 59, 5 min.: Initially, numerous naturally fluorescent bodies, white or yellowish-white, of high intensity. Widespread delineation of surface polygonal pattern by blue fluorescence, from moderate to high intensity. Esculin not detectable in hepatic cells. Many interruptions in surface pattern, but suggestive blindly ending projections almost invariably plunge into substance of liver, and connect with other canaliculi at first sub-surface level. These in turn connect with still more deeply lying canaliculi, which at second sub-surface level may exhibit a polygonal pattern analogous to that on surface. Canaliculi at first sub-surface level delineated at moderate intensity, and frequently exhibit a misleading picture of "axial" canaliculi with lateral "branches". At end, extrahepatic bile passages and gall bladder exhibit intense esculin fluorescence.

No. 56, 15 min.: Initially a brilliant display of naturally fluorescent bodies. Surface polygonal pattern sharply delineated by electric blue fluorescence, in striking contrast to dark background. Esculin not detectable in hepatic cells. Intensity of surface pattern exhibits regional variation, and canalicular delineation in underlying laminae roughly proportional in any given area. Canaliculi at first sub-surface level sharply delineated, and repeatedly

TABLE IV

Observations upon White Mice Sacrificed at Intervals following Intraperitoneal Injection of Esculin 30 mg. per kg.[1]

NO.	WEIGHT	INTERVAL	INTENSITY OF ESCULIN IN BILE CANALICULI[2]			
			High	Moderate	Low	Very low
Series A						
	gm.	min.				
131	19.6	5	¾	¼	—	—
132	18.6	10	Few	½	½	—
133	20.1	15	—	Few[3]	½	½
134	20.8	20	—	—	⅓	⅔
135	19.3	20	—	Rare	Few	Most
136	19.3	25	—	—	—	—
137	22.6	30	Few	½	½	—
138[4]	19.5	45	—	—	—	—
Series B						
173	17.0	5	—	⅛	⅞	—
174	18.0	10	—	Rare	½	½
175	17.5	15	⅕	⅖	⅖	—
176	18.0	20	—	—	⅛	¾[5]
177	17.5	25	—	Rare	½	½
178	16.3	30	—	—	Rare	All
179	16.1	40	—	—	—	Few
180	14.8	60	—	—	—	—
181	17.9	60	—	—	—	(Few)[6]
182	16.0	80	—	—	—	—

[1] At end of observations upon liver, esculin noted to be essentially absent from gall bladder but present in extrahepatic bile passages at low (no. 174) or moderate intensity (nos. 131 and 173); in both bladder and passages at low (nos. 132, 134 and 138) or moderate intensity (nos. 135, 137 and 140); in bladder at very low, passages at low (no. 178)—in bladder at low, passages at very low (nos. 179 and 181) or moderate (no. 133)—and in bladder at moderate, passages at very low (nos. 139, 177, 180 and 182), low (nos. 136 and 175) or high intensity (no. 176).

[2] Esculin not detectable in cytoplasm of hepatic cells in any of the animals, with following exceptions: No. 133—some questionable pictures obtained, suggestive at best. Nos. 173 and 174—in a number of instances a curious picture of apparent esculin fluorescence immediately adjacent to bile canaliculi, involving only a limited portion of the hepatic cells concerned, and in no case an entire cell territory.

[3] In one small area several instances of delicate "beading" noted in most superficial canaliculi.

[4] Mice nos. 139 (19.5 gm.) and 140 (18.2 gm.), sacrificed at 60 and 75 minutes respectively, likewise showed no trace of esculin.

[5] Esculin not detectable in bile canaliculi in approximately ¼ of area surveyed.

[6] In initial two-minute survey, no bile canaliculi visualized in left lower lobe. Left upper lobe then surveyed, ¾ of canaliculi delineated with extreme delicacy. Left lower lobe then resurveyed, and canaliculi found to be delineated with extreme delicacy in occasional areas.

connect with canaliculi in surface pattern and at second sub-surface level. No blindly ending intercellular branches. Continuous polygonal meshworks, in plane of focus, frequently encountered at second sub-surface level.

TABLE V

Observations upon White Mice Sacrificed at Intervals following Intraperitoneal Injection of Thioflavine S 30 mg. per kg.[1]

NO.	WEIGHT	INTERVAL	INTENSITY OF THIOFLAVINE S							
			In Bile Canaliculi				In Hepatic Cells[2]			
			High	Moderate	Low	Very low	High	Moderate	Low	Very low
Series A										
	gm.	min.								
141	23.6	5	½	¼	¼	—	¼	½	⅛[3]	—
142	21.3	10	All	—	—	—	Rare	¾	¼	—
143	20.6	15	¾	¼	—	—	¼[4]	½	¼	—
144	23.1	20	¾	¼	—	—	—	¼	¾	—
145	22.2	25	¾	¼	—	—	Rare	¾	¼	—
146	20.2	30	½	½	—	—	—	¼	¾	—
147	23.5	40	½	½	—	—	—	¼	¾	—
148	21.6	50	¼	¾	Few	—	—	¼	½	¼
149	22.9	60	¼	¾	—	—	—	¼	¾	Few
Series B										
150	23.2	60	⅛	⅞	—	—	—	—	⅞	⅛
151	25.5	75	⅛	⅞	—	—	—	—	¾	¼
152	26.6	90	—	⅞	⅛	—	—	—	¾	¼
153	25.7	110	—	⅞	⅛	—	—	—	¾	¼
154	24.2	130	—	¾[5]	¼	—	—	—	¾	¼
155	27.7	150	—	⅓	⅔	—	—	—	¼	¾
156	26.8	180	—	Rare	¾	¼	—	—	¼	¾[6]
157[7]	22.0	210	—	—	¼	¾	—	—	—	—
158[8]	20.1	240	—	—	¼[9]	¾[9]	—	—	—	—
Series C										
159	27.3	240	—	Rare	¾	¼	—	—	—	½
160	26.7	285	—	—	¾	¼	—	—	—	?[10]
161[11]	26.3	330	—	—	—	—	—	—	—	—
162[12]	24.0	375	—	—	⅛	⅞	—	—	—	—
163[13]	24.6	420	—	—	Rare	All	—	—	—	—
Series D										
		hr.								
164	15.8	7	—	—	All	—	—	—	—	—
165	15.0	8	—	—	—	All[14]	—	—	—	—
166	18.2	9	—	—	—	All[14]	—	—	—	—
167	17.6	10	—	—	½	½	—	—	—	—
168	17.9	11	—	—	—	All[14]	—	—	—	—
169	18.6	12	—	—	—	All[14]	—	—	—	—
Series E										
170	17.8	15	—	—	—	All[15]	—	—	—	—
171	18.6	18	—	—	—	All[14]	—	—	—	—
172	16.8	24	—	—	—	¼[14]	—	—	—	—

Studies with thioflavine S

The normal sequence of events in the mammalian liver, following the intraperitoneal injection of thioflavine S 1:1,000 in a dosage of 30 mg. per kg., is illustrated by the data summarized in Table V. As with fluorescein and esculin, the bile canaliculi always appeared as a continuous system, and no instance was encountered of "sprouting" or of intracellular vacuole formation. The following more detailed observations were made upon white mice (21.5–27 gm.), sacrificed at varying intervals following intravenous injection of thioflavine S 1:1,000 at uniform dosage of 30 mg. per kg. The delay between sacrifice and visualization of the liver varied between 2 min. 50 sec. and 4 min. 45 sec., and the length of the observation period varied between 7 and 15 minutes. All observations were made with the 90× lens upon the ventral surface of the left lower lobe. As in the frog, the initially sharp pictures of the bile canaliculi rapidly decrease in fluorescent intensity and clarity when any microscopic field is held under continuous observation, and disappear within a few minutes.

No. 63, 5 min.: Bile canaliculi in surface polygonal pattern and at first sub-surface level strikingly delineated, moderate to high intensity, rich blue or (at highest intensity) whitish-blue color. Can penetrate occasionally to second sub-surface level, where an essentially polygonal meshwork (in plane of focus) is encountered. Unusually large number of sinusoids

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[1] At end of observations upon liver, thioflavine S noted to be present in gall bladder at low, in extrahepatic bile passages at very low intensity in no. 141; in both bladder and passages at low (nos. 158, 171 and 172), moderate (nos. 142, 143, 144, 147, 149, 150, 154 and 162) or high intensity (nos. 152, 153, 155, 160 and 167); in bladder at low, passages at low (nos. 164, 169 and 170) or moderate (nos. 145 and 146)—in bladder at moderate, passages at low (nos. 166 and 168)—in bladder at high, passages at very low (no. 163), low (no. 156) or moderate intensity (nos. 148, 151, 157, 159 and 165). The dye could not be detected in the bladder or passages in no. 161.

[2] Nuclei of hepatic cells apparently routinely spared by the dye in all animals (however, see footnote 4).

[3] Dye not detectable in cytoplasm of remaining ($\frac{1}{4}$) hepatic cells. Cells exhibit marked regional variation in fluorescent intensity.

[4] In one small area cytoplasmic fluorescence unusually high and chalky-white in color; nuclei can not be visualized.

[5] In a number of instances canaliculi noted to exhibit several short, delicate, lateral branches, same intensity as parent canaliculi.

[6] In a few instances distinctive fluorescence of dye not detectable with assurance.

[7] After first 2 minutes, second (left upper) lobe surveyed. Canaliculi $\frac{1}{2}$ low, $\frac{1}{2}$ very low intensity. No trace of dye detectable in cells.

[8] After first 2 minutes, second (left upper) lobe surveyed. Canaliculi $\frac{1}{2}$ low, $\frac{1}{2}$ very low intensity. No trace of dye detectable in cells.

[9] Considerable regional variation in intensity of canalicular fluorescence.

[10] Presence of dye suggestive but questionable in majority of cells.

[11] After first 2 minutes, second (left upper) lobe surveyed, and likewise showed no trace of the dye.

[12] After first 2 minutes, second (left upper) lobe surveyed; canaliculi $\frac{1}{4}$ low, $\frac{3}{4}$ very low intensity. No trace of dye detectable in cells.

[13] After first 2 minutes, second (left upper) lobe surveyed, with essentially same findings.

[14] Delineated with extreme delicacy. In nos. 171 and 172 frequently suggestive at best.

[15] About half of them delineated with extreme delicacy.

achieve an immediately subcapsular position, and regions of continuous subcapsular sheets or laminae of hepatic cells more limited in both numbers and extent than customarily observed. At first sub-surface level picture one of "axial" canaliculi with many lateral "branches", which freely connect with surface polygonal pattern and with canaliculi at second sub-surface level. Clearly evident that many canaliculi, as they extend from surface polygonal pattern to first sub-surface level, course between surface of laminae and adjacent sinusoidal walls, in immediate contact with latter. Cytoplasm of hepatic cells exhibits blue induced fluorescence, low to moderate intensity. Nuclei routinely spared by the dye, appear as non-fluorescent areas.

No. 62, 10 min.: Canaliculi delicately delineated, moderate to high intensity. Picture as complete and striking as ever obtained. Surface polygonal pattern well displayed, and its many connections with canaliculi at first sub-surface level very easily traced. Clear pictures of "axial" canaliculi and their "branches" frequently observed at first sub-surface level. An essentially polygonal pattern, analogous to that on surface, visualized at times at second sub-surface level. For most part induced fluorescence of hepatic cells is suppressed (very low or low intensity, seldom moderate).

No. 64, 20 min.: Canalicular delineation apparently complete, moderate or high intensity. In best areas can penetrate readily from surface polygonal pattern to picture of "axial" canaliculi and their "branches" at first sub-surface level, and in turn to rich polygonal meshwork (in plane of focus) at second sub-surface level. Canaliculi at all levels freely interconnect, and no instance of a blindly ending "intercellular branch" encountered. Cytoplasm of hepatic cells shows blue induced fluorescence, usually at moderate, occasionally at low or high intensity. Nuclei regularly spared by the dye, no other details of cellular structure can be visualized.

No. 65, 30 min.: Canaliculi delineated predominantly at low, sometimes at very low or moderate intensity. Cytoplasm of hepatic cells exhibits blue induced fluorescence, very low or low intensity. Nuclei routinely non-fluorescent. Surface polygonal pattern shows many interruptions; at such points its "free" branches almost invariably seen to plunge into substance of liver, establishing connections with canaliculi at first sub-surface level. Very frequently such extensions can be seen joining so-called "axial" canaliculi of first sub-surface level. In some instances can visualize with surprising clarity essentially polygonal character of meshwork throughout. In horizontal plane at surface, in vertical plane at first sub-surface level, and again in horizontal plane at second sub-surface level. In numerous instances a vertically "zig-zag" course of "axial" canaliculi at first sub-surface level very clearly evident.

Observations upon the following two white mice (34 and 28 gm.), which received thioflavine S intravenously at a dosage of 50 mg. per kg., are described in some detail in so far as they relate to the architecture of the bile canalicular system. Observations were made with the 90× lens upon the ventral surface of the left lower lobe, and the delay between sacrifice and visualization of the liver was 2 min. 35 sec. and 2 min. 30 sec. respectively.

No. 71, 15 min.: Canaliculi completely delineated, predominantly at high intensity. Sharpness of delineation affords unusually good opportunity for detailed study of canaliculi at surface and first sub-surface level. Picture so brilliant that it is difficult to penetrate to deeper levels. Multiple connections between surface polygonal pattern and canaliculi at first sub-surface level can be traced with ease in very many instances. Most characteristic type of connection is from a branching or junction point of surface polygonal pattern, and

plunging almost straight down to "axial" canaliculus of lamina at first sub-surface level. Over-all picture one of a continuous, never-ending, polygonal meshwork of canalicular system. However, in several instances encounter what appear to be short, blindly ending "intercellular branches" at first sub-surface level.

No. 74, 30 min.: Canaliculi strikingly displayed everywhere in surface polygonal pattern and at first sub-surface level, predominantly at high intensity. Can frequently penetrate with relative ease to second sub-surface level, where unequivocal pictures of essentially continuous polygonal meshwork in plane of focus are the usual finding. Interconnections between surface polygonal pattern and canaliculi at first, and latter in turn with those at second sub-surface level, so rich and so readily traced as to give most convincing picture of bile canalicular system as having an essentially continuous, never-ending, polygonal meshwork as its basic architecture. In very many instances an extension from a junction point in the surface polygonal pattern plunges directly down to an "axial" canaliculus at first sub-surface level. In other instances a lateral extension from surface polygonal pattern curves gently over edge of a lamina and then plunges vertically, so far as one can tell on surface of lamina and between it and sinusoidal wall. For relatively few branches of canalicular system is it impossible to demonstrate a connection with remainder of system, and in no instance can possibility of such a connection be denied with assurance. Can at times penetrate to third and fourth sub-surface levels, and sharpest pictures obtained indicate that basic pattern and continuity of canaliculi are same at deeper as at more superficial levels.

Supplementary observations were made upon the liver in single specimens of the rabbit and monkey, sacrificed at 26 and 56 minutes respectively after completion of the intravenous injection of thioflavine S in a dosage of c. 50 mg. per kg. The findings were entirely consistent with those in the mouse and rat.

Rabbit no. 3, female, 3.64 kg.:

Between 0 and 8 minutes injected intravenously (ear) 44.7 ml. of thioflavine S 1:250. At 34 minutes sacrificed by intravenous injection of air. Liver exposed and ventral surface surveyed *in situ* between 44 and 49 minutes. Liver then removed from body, and ventral surface resurveyed between 58 and 93 minutes. All observations with 90× lens. A single description will suffice.

Bile canaliculi everywhere delineated, majority at moderate, minority at low, occasionally at high intensity. Canalicular apparatus appears to be a continuous, essentially polygonal meshwork at surface and in substance of liver. No blindly ending branches encountered. Caliber of individual canaliculi and size of polygons in meshwork essentially identical with those in mouse and rat. Cytoplasm of hepatic cells exhibits blue induced fluorescence of low intensity; nuclei visualized in minority of instances as non-fluorescent areas.

In course of survey noted many instances of "beading" of canaliculi and of delicate "sprouts" or "twigs", quite variable in number and length, arising from canaliculi; latter interpreted as abnormal and equivalent to those described by Hirt *et al.* (2).

Monkey no. 1, Macacus rhesus, adult female, 6.15 kg.:

Hysterectomy some time previously, apparently in excellent health. Between 0 and 22 minutes injected intravenously (veins of leg) c. 77 ml. of thioflavine S 1:250. Sacrificed by intravenous injection of air at 78 minutes. Liver removed from body *in toto*, after first clamp-

ing ductus choledochus and main tributaries (identified under ultraviolet light, essentially white in color, high intensity). Between 97 and 122 minutes surveyed surface of all lobes, for most part with 90×, checked occasionally with 23× lens.

In most fields bile canaliculi delineated, at low or very low intensity, as an apparently continuous polygonal meshwork. Caliber of individual canaliculi and size of polygons essentially identical with those in mouse, rat and rabbit. Cytoplasm of most hepatic cells devoid of recognizable induced fluorescence attributable to dye; nuclei not visualized anywhere. Scattered over all surfaces, occurring singly or in small groups, many hepatic cells exhibiting moderate or high fluorescent intensity of cytoplasm; nuclei not visualized.

The normal mechanism of biliary excretion

These studies upon mammals, sacrificed at varying intervals—throughout the excretory process—following injection of various compounds, and brought under observation as quickly as possible, have demonstrated to our entire satisfaction that the normal mechanism of biliary excretion is fundamentally the same in the mammal as in the frog. As illustrated by the data summarized in Tables I–V, there is a marked difference in the time required for the complete elimination from the liver of fluorescein, esculin and thioflavine S, when administered in the same dosage and by the same route. Furthermore, in the study of large numbers of animals over an extended period, we have observed with each of the compounds a considerable variability in the time relationships of the excretory process. Nevertheless, the process is in all cases an orderly one, and follows a similar course.

The bile canaliculi at all times maintain their identity as discrete tubes, with walls of definite and essentially linear contours, and with no suggestion of the extraordinary plasticity implicit in the "sprouting" phenomenon described by Hirt *et al.* Compared with their delicate delineation in the early and late stages of excretion, the bile canaliculi are on the whole significantly larger in caliber at the peak of excretion, and this is entirely comprehensible on physiological grounds. In the terminal stages of excretion the delineation of the canaliculi is so exquisitely delicate as severely to test both the equipment and the observer. However, so long as they are delineated they persist as a continuous system, without detectable interruptions in their course.

The formation, within the hepatic cells, of vacuoles containing fluorescein, esculin or thioflavine S simply does not occur under normal conditions. If it did, to anything like the extent claimed by Hirt *et al.*, the liver would normally exhibit for these compounds a storage phase of such large proportions that it could readily be detected by other means. Thus far, no evidence for such a phase has been forthcoming.

Architecture of the bile canalicular system

When sharply delineated by fluorescent compounds, the bile canaliculi in the surface polygonal pattern are everywhere continuous, either with each other or with canaliculi in the underlying laminae. Whenever the canalicular

system can be sharply visualized, such connections can be freely traced. The pictures are so convincing that one inevitably doubts a break in the continuity of the system, even when the existence of such a break can not be positively excluded in a given instance. Similar continuity has been traced innumerable times between the canaliculi at the first and second, second and third, third and fourth, and fourth and fifth sub-surface levels. This is about as far as we can penetrate into the substance of the liver with our present equipment. Also, even this degree of penetration can only be achieved at the expense of a progressive loss in the fluorescent intensity of the pictures obtained.

The various sub-surface levels referred to above represent much more than a rough approximation by the observer. As one focuses more and more deeply below the surface of the liver, the picture of the bile canalicular system changes in a way which we have found to be as characteristic as it was unexpected.

At the first sub-surface level the surface polygonal pattern is usually replaced by a picture which frequently simulates, at first glance, that found typically in the frog, i.e., axial canaliculi provided with numerous intercellular branches. However, careful study at this level shows clearly that these "intercellular" branches are almost invariably continuous with canaliculi at the same or other levels. Furthermore, the "axial" canaliculi prove to be merely a partial representation of a continuous polygonal meshwork, which is oriented in a plane roughly perpendicular to the surface of the liver. The true character of this meshwork can best be appreciated when the underlying lamina forms a more or less acute angle, rather than a right angle, with the subcapsular lamina.

At the second sub-surface level one again encounters, with surprising frequency, a polygonal pattern oriented approximately in the same horizontal plane as that on the surface. The pictures certainly suggest very forcibly that the laminae at the first sub-surface level have changed their course by about 90 degrees. Here again, the canaliculi are freely continuous with those on the same or other levels. In general, the picture at the third sub-surface level is analogous to that at the first; the picture at the fourth sub-surface level is analogous to that at the second; and the picture at the fifth sub-surface level is analogous to that at the first and third.

In other words, the mammalian liver frequently exhibits—as one focuses progressively more deeply into its substance—a fairly regular alternation in plane, but not in character, of a polygonal meshwork of bile canaliculi, which appear to be everywhere continuous with each other. In our experience the exceptions to continuity are extremely few, and even then at times very questionable. We have become convinced that the fundamental architecture of the bile canalicular system is that of a continuous, never-ending, polygonal meshwork. Intercellular branches, ending blindly—which are so characteristic of the amphibian liver—are apparently of such infrequent occurrence in the normal mammal as to be insignificant.

By different means, we have arrived at essentially the same conclusion regarding the bile canalicular system as Elias (18), who in recent years has done so much to clarify our understanding of the structure of the mammalian liver. His major thesis (19, 20), that the hepatic laminae in mammals are typically one hepatic cell in thickness, we have abundantly confirmed by our methods.

The concept of a refractory period in the secretory activity of the liver

As mentioned above, Hirt *et al.*'s (2) concept of a refractory period in the secretory activity of the liver has been exhaustively tested in the frog, with

TABLE VI

Observations upon White Mice Receiving Two Intraperitoneal Injections of Fluorescein 30 mg. per kg. Separated by Varying Intervals, and Sacrificed 10 Minutes After the Second Injection[1]

NO.	WEIGHT	INTERVAL BETWEEN INJECTIONS	INTENSITY OF FLUORESCEIN IN BILE CANALICULI		
			High	Moderate	Low
		Series A			
	gm.	min.			
92	28.1	10	⅗	⅓	Rare
93	29.1	20	½	½	Few
94	29.8	30	Most	Few	—
95	34.3	45	⅗	⅓	—
96	29.8	60	¾	¼	Rare
97	29.0	75	⅗	⅓	—
98	20.3	90	¾	¼	—
		Series B			
99	26.9	70	⅗	⅓	Rare
100	30.6	80	⅔	⅓	⅙
101	40.4	90	½	½	Few
102	37.2	100	⅙	⅓	⅓
103	38.6	110	⅙	⅗	⅙
104	37.1	120	Most	Few	—
105	36.1	135	¼	¾	Rare
106	30.1	150	½	½	—

[1] Fluorescein was detectable in the cytoplasm of the hepatic cells in all animals at low or very low intensity (in nos. 92, 93, 95, 96, 97, 99, 101, 102 and 105 in about equal proportions; in nos. 94, 98, 103, 104 and 106 more frequently at low (¾) than at very low intensity; in no. 100 more frequently at very low (¾) than at low intensity).

consistently negative results. The conditions for testing it in mammals are essentially the same as in amphibia: 1. two injections of a given substance at standard dosage under standard conditions, separated by varying intervals; 2. sacrifice, followed promptly by exposure and study of the liver, at the end of an experimentally acceptable minimum period following the second injection. In this way, any abnormalities attributable to such obvious factors as

anesthesia, prolonged observation of the liver, etc. may be avoided in so far as possible.

After study of the available data, it was decided to use white mice; to give them two intraperitoneal injections of fluorescein, each at a dosage of 30 mg. per kg., separated by graded intervals between 10 minutes and 2½ hours; and to sacrifice them for study 10 minutes after the second injection. Examination of Table I shows that, following a single intraperitoneal injection of fluorescein at this dosage, the liver is at or near the peak of excretion in 10 minutes, and that elimination of the dye from the liver is complete in 1½ hours or less. Under the circumstances, in the absence of any appreciable refractoriness on the part of the liver to the dye offered by the second injection, all of the animals in the series would be expected to exhibit a fairly consistent response. In terms of bile canalicular delineation, they should all show a picture comparable with that found 10 minutes after a single injection.

The results of the study are summarized in Table VI, and give an unequivocal answer. Canalicular delineation by the dye is in all instances complete, and on the whole at moderate or high intensity. The findings are inconsistent with the occurrence in the mammalian liver, with respect to fluorescein, of even a brief refractory period, let alone one of several hours' duration as claimed by Hirt *et al.* Under their abnormal experimental conditions, the failure of the liver to respond to a second injection of fluorescein is meaningless as far as the normal function of the liver is concerned.

Studies with acriflavine

As in the frog, these studies did not contribute to our understanding of the mechanism of biliary excretion, and discussion of the observations will be limited to the following brief comments. The entire sequence of events—as reported by Hirt *et al.* (2) for trypaflavin—can be induced, though far more delicately than they observed it, in the liver of a white mouse (60 mg. per kg., sacrificed 45 minutes later) directly exposed to rather intense ultraviolet radiation under a quartz mercury vapor lamp. Bile canalicular delineation by the dye has not been observed. Markedly toxic effects of acriflavine may be quite evident in mice c. 13 hours following injection of the dye in higher dosage (90–270 mg. per kg.). Even in such animals nuclear staining could not be visualized.

Attempts to effect retrograde injection of the bile canaliculi

Up to now our efforts to effect a retrograde injection of the bile canaliculi with fluorescein have met with no success. It appears that the biliary tract is easily ruptured at points distal to the canaliculi themselves; and a partial vascular injection of the dye could readily account for all of our findings thus far in both rat and rabbit. It is interesting to note that Franke and Sylla's (10) efforts in the frog were likewise entirely unsuccessful.

Abnormal phenomena encountered under conditions of prolonged observation, anesthesia and anoxia

It has been adequately demonstrated that in the livers of mammals sacrificed at varying intervals—throughout the excretory process—following the injection of fluorescein, esculin and thioflavine S, and promptly brought under microscopic observation, the bile canalicular system and the hepatic cells are consistently devoid of the bizarre phenomena reported and interpreted as normal by Hirt *et al.* (2). These include extensive "sprouting" of small vacuoles from bile canaliculi, widespread intracellular vacuole formation, discontinuity of bile canaliculi, and the formation of so-called "lakes" of fluorescein, etc. However, all of these aberrant phenomena have been observed in the mammal, as in the frog, under conditions sufficiently abnormal to make it obvious that they play no rôle in the normal mechanism of biliary excretion.

The phenomena frequently develop, in varying degree, when the livers of freshly sacrificed mammals are subjected to prolonged observation under the fluorescence microscope. The same is true in mice and rats maintained under anesthesia. Minor abnormalities (i.e., a more or less delicately "beaded" appearance) of the canaliculi, develop at times rather quickly, in a matter of several minutes. "Sprouting" and intracellular vacuole formation usually appear only after a considerable delay. However, the new development of short, delicate, blindly ending branches from the canaliculi has been noted to occur after a relatively brief interval, particularly in mice sacrificed following the injection of thioflavine S. So far as we can tell, these branches may be either "intercellular" or "intracellular" in location. The picture is an unusual one, and the evidence is overwhelming that these branches constitute a distinctly abnormal phenomenon.

The vacuoles which develop in the cytoplasm of the hepatic cells, and are delineated by their content of fluorescein, esculin or thioflavine S, appear at times to be connected to adjacent bile canaliculi by delicate channels, of variable length and caliber, and similarly delineated. In very numerous instances, however, no such connection can be visualized; and some of the pictures obtained strongly suggest that, for many of the vacuoles at least, such a connection never existed.

The following protocol illustrates the development of grossly abnormal phenomena in the liver of a white mouse, subjected to progressive anoxia for 10 minutes following injection of fluorescein at a dosage of 60 mg. per kg., then sacrificed and promptly brought under observation.

No. 130, 21.7 gm.:

0 Injected intraperitoneally 0.33 ml. of fluorescein 1:250. Animal promptly placed in 100 ml. lusteroid tube, which was tightly stoppered. Tube contained perforated capsule (capacity c. 6 ml.) filled with soda lime for absorption of CO_2.

10′0″ Released from tube, rather active as soon as removed; sacrificed, liver exposed.

12′10″ Survey of ventral surface of left lobe lobe, 55× lens. Bile canaliculi everywhere delineated at high (2/3), moderate (1/3) or occasionally low intensity. With few exceptions fluorescein detectable in cytoplasm of hepatic cells, low (2/3) or very low intensity (1/3). Majority of canaliculi and hepatic cells exhibit no appreciable abnormality. However, principally in most superficial laminae, to some extent in those just below surface, observe the following: 1. Very many instances of more or less delicate "beading" of canaliculi. 2. Many instances of canaliculi exhibiting from several to many short, stubby, lateral branches, variable in length and caliber, and apparently ending blindly. 3. Many instances of canaliculi exhibiting a whole series of small (variable in size), spherical protuberances or vacuoles, delineated at essentially same intensity and color as parent canaliculus. Very closely applied to canaliculus, impossible to determine whether they are connected thereto by delicate channels, or represent simple outpocketings of canalicular wall. 4. Many instances of spherical, fluorescein-containing vacuoles within hepatic cells. Small and medium size, occasionally large, intensity and color similar to adjacent canaliculi. Located peripherally in cytoplasm, central portion of cells free of them. Vacuoles at times appear to be connected to canaliculi by delicate fluorescein-containing channels; usually such channels can not be visualized. Canaliculi in areas of extensive vacuole formation frequently exhibit apparent discontinuity, varying in degree, in their delineation by the dye. Many times vacuoles involve only an isolated cell, but frequently involve, 2, 3 or more adjacent cells, and sometimes involve whole areas to give an extraordinary display of Hirt *et al.*'s phase of intracellular vacuole formation. Cytoplasm of cells concerned may exhibit (a) no detectable trace of the dye; (b) usual picture of fluorescein fluorescence, at low or very low intensity and predominantly yellow in color; (c) fluorescence of low intensity and a soft yellowish-green in color, with green dominant; (d) fluorescence of moderate or even high intensity and predominantly yellow in color. 5. Occasional instances, in hepatic cells scattered irregularly over the surface, of one, two or several rather large (nuclear size or larger), spherical vacuoles exhibiting a diffuse, soft green fluorescence of low intensity. These vacuoles appear quite different from those described in 4 above, and unlike them frequently occupy more central portion of cells. Rarely occur in two or more adjacent cells.—These abnormal pictures did not appear to develop in extent or degree up to the end of the observation period at *15′10″*.

15′30″ Survey of ventral surface of left upper lobe, 55× lens. Findings essentially the same as in left lower lobe, but fewer instances of intracellular vacuole formation. Survey completed at *18′30″*.

19′0″ Resurvey of ventral surface of left lower lobe, principally with 55× lens. Areas of intracellular vacuole formation now so extensive that this aspect of picture had almost certainly developed in interim. Also, large vacuoles (nuclear size or larger), containing fluorescein at moderate or high intensity, now apparently more numerous than before. Earlier observation of channels connecting minority of vacuoles (of varying size) to adjacent canaliculi confirmed with more assurance. In fields held under continuous observation for short periods, no appreciable change noted in character, size or color of vacuoles, canaliculi, etc. Wide resurvey of surface toward close of observations indicates that picture of intracellular vacuole formation has progressed still further during period.

33′ Observations terminated. At end, noted presence of fluorescein at high intensity in extrahepatic bile passages and gall bladder.

Earlier extensive studies in the frog (1) have made all too obvious the abnormal character of the bizarre phenomena described by Hirt *et al.* in the later stages of the excretory process. This view has been fully confirmed in the mammal by less extensive studies, which have not been carried to greater length since our primary concern has been with the normal mechanism of biliary excretion. However, the nature of these phenomena is so extraordinary that they clearly merit further detailed investigation.

DISCUSSION

The principles and technic of fluorescence microscopy have been fully described by others (7, 15, 21, 22), and have found wide application in biology and medicine.

While our studies of the amphibian liver are largely based upon *in vivo* observations in pithed specimens, in mammals we have relied primarily upon examination of the liver as soon as possible after death. We are well aware of Ellinger's statement that "the fluorescent image due to fluorescin changes very rapidly after death" (15). We have confirmed this statement, and have noted a striking alteration in the luminosity (reduced) and color (shifted from green toward yellow) of fluorescein in the cytoplasm of the hepatic cells when an anesthetized mammal is sacrificed. However, we have been unable to detect, in the immediately postmortem period, any concomitant rapid change in the pictures of the bile canalicular system. We are likewise well aware of Ellinger's dictum that "the study of a physiological process cannot be carried out with the dead organ" (15). In the present instance we would counter this by noting that no more abnormal pictures of the mechanism of biliary excretion, in both amphibia and mammals, have been reported than those obtained by Hirt *et al.* (2) under anesthesia, and using all the refinements of intravital fluorescence microscopy. We have been practically forced to use freshly sacrificed mammals, and by so doing have arrived at what we believe is an essentially valid account of the normal excretory process.

The chemical structure of fluorescein, esculin (23, 24), acriflavine and proflavine is well established. The structure of thioflavine S, on the other hand, is not exactly known, and the information given in Figure 1 is derived from the Colour Index (25). The nature of this compound is discussed at some length in the recent paper by Moses *et al.* (26). The "trypaflavin" of Hirt *et al.* (2) was presumably (25) the methylated component (formula I, Fig. 1) of our sample of acriflavine. All of the fluorescent compounds which we have studied appear to be essentially non-toxic in the dosage routinely used, 30 mg. per kg., which is a very small fraction of the lethal dose of fluorescein as reported by Jodlbauer and Busck (27).

The striking photosensitivity of thioflavine S is part of the larger picture of photodynamic effects associated with fluorescent compounds, upon which there

has developed an abundant literature (6, 28–30) since Raab's (31) discovery of the phenomenon with the acridine dyes. It is noteworthy that the rapid quenching of the fluorescence of thioflavine S in the bile canalicular system is not accompanied by any similar effect upon the dye present in the cytoplasm of the associated hepatic cells. Schlegel and his associates (26, 32, 33) have recently made extensive use of thioflavine S for the delineation of functionally active blood vessels.

Bradley (34) has reported that fluorescein is largely bound to the plasma proteins, and that only from 10 to 20 per cent of the dye can pass through a cellophane membrane. He and his associates studied the disappearance of the dye from the blood in normal subjects and in patients with various hepatic disorders. They found that it is removed less rapidly than bromsulphalein, at rates of approximately 4 to 5 per cent of the plasma concentration per minute in the normal, and at rates from 2 to 3 per cent per minute in the presence of liver disease. Bradley states that 50 to 65 per cent of the dye is excreted in the urine, and further reports that bromsulphalein and fluorescein are removed from the blood independently, without evidence of mutual interference when both are present.

Particular interest attaches to the naturally fluorescent bodies which are regularly present in the cells lining the hepatic sinusoids in both amphibia and mammals, and the fluorescence of which is rapidly extinguished by exposure to ultraviolet light. Franke and Sylla (10) first properly described the location of these bodies, which have been interpreted by others (35–37) as one of the loci of vitamin A, characteristic of which is a green, fading fluorescence. Although our observations are otherwise in agreeemnt with those previously reported, we have yet to detect a green component in the fluorescence of the bodies, the color of which is predominantly yellow. In addition to the *in vivo* studies previously mentioned (1, 2, 10, 16), particular reference is made to the studies of the natural fluorescence of the liver (and other organs) in post-mortem material by Hamperl (38) and Sjöstrand (39).

The excretion of fluorescein by the frog's liver, as observed *in vivo*, was first described by Ellinger and Hirt (21, 40), who provided an excellent illustration of the normal bile canalicular system as delineated by the dye at or near the peak of excretion. The work of Hartoch (41) did little to clarify the problem, but the subsequent investigations by Franke and Sylla of the mechanism of biliary excretion in amphibia (10) and mammals (16) are highly contributory, and merit careful study. Their reports are documented by the first successful fluorescence microphotographs of phenomena in the living animal, and their technic was further developed by Franke (42). The work of Hirt, Ansorge and Markstahler (2–5) has already been described in sufficient detail. A discussion of *in vivo* studies of fluorescein excretion under various abnormal conditions—biliary obstruction (2, 10, 41), ligation of portal vein (2), drug poisoning (10,

16, 41) and X-radiation (43)—is beyond the scope of this paper. Our observations consistently refute the idea of the occurrence of intracellular branches of the bile canaliculi under normal physiological conditions (44).

It was earlier stated that there was no evidence for the existence of any route, other than through the cytoplasm of the hepatic cells, for the transfer of material from the blood in the sinusoids to the bile canaliculi. On the basis of the present study, this statement must now be withdrawn. In the frog, intercellular branches of the axial canaliculi have frequently been seen to achieve immediate contact with the sinusoidal wall. In the mammal, bile canaliculi have in numerous instances been observed to course between the walls of the sinusoids and the adjacent hepatic laminae. In both forms, anatomical arrangements are present which would permit direct transfer of substances from the sinusoids to the bile canaliculi, or vice versa, without passage through hepatic cell cytoplasm. In this connection, reference is made to the monograph of Pavel (45). Our study has offered no information as to whether such transfer actually occurs.

In routine histological preparations of the mammalian and particularly of the human liver, in health and disease, the fragmentary pictures of the bile canalicular system observed frequently lend themselves to more than one interpretation as far as architecture is concerned. In many instances they suggest an arrangement more or less at variance with the concept of a continuous polygonal meshwork, and may even simulate the amphibian pattern of axial canaliculi with their intercellular branches. However, we are convinced that the concept is fundamentally a sound one. The detailed analysis by Elias (18) of the three-dimensional aspects of hepatic and canalicular architecture has done much to clarify the problem, and merits the most careful study. Our own conclusions are based primarily upon observations in the white mouse, and the extent of their applicability to the human liver must await further study.

Following intraperitoneal or intravenous injection in mammals, the rapidity of complete delineation of the bile canaliculi by fluorescein, esculin or thioflavine S, and of uniform cytoplasmic staining by fluorescein or thioflavine S, makes it appear that the great bulk of the hepatic cells are regularly in contact with an active sinusoidal circulation. The consistency with which such pictures are obtained must be taken into account in any consideration of the problem of intermittence of blood flow in the hepatic sinusoids (46–50).

As discussed above, a picture of more or less widespread intracellular vacuole formation may develop in the liver rather rapidly under abnormal conditions. Under the fluorescence microscope these vacuoles are sharply delineated by their content of fluorescein, esculin or thioflavine S, all of which are water-soluble compounds. If any single factor plays an essential rôle in the development of these vacuoles, anoxia seems the most likely possibility. The pictures

obtained appear to have a great deal in common with those of watery ("anoxic") vacuolation of the liver described by others—in both laboratory mammals and man—under conditions of perfusion, decompression, anoxia, asphyxia, hemorrhage, carbon monoxide poisoning, etc. (51–58). The conclusion is probably justified that all of these pictures of watery vacuole formation are manifestations of the same fundamental process.

SUMMARY

Earlier observations upon the normal mechanism of biliary excretion of fluorescein in the frog have been confirmed and extended by the use of esculin and thioflavine S.

Studies upon mammals, sacrificed at varying intervals following injection of fluorescein, esculin and thioflavine S, have clearly demonstrated that the normal mechanism of biliary excretion is fundamentally the same as in the frog. At the peak of excretion all bile canaliculi are sharply delineated at high fluorescent intensity, which exhibits a progressive diminution to non-detectable levels as the compounds are eliminated from the liver. The canaliculi always appear as a continuous system, without interruptions in their course, and maintain their identity as discrete tubes.

In the mammal, as in the frog, there is no evidence for the occurrence of a refractory period, with respect to fluorescein excretion, in the secretory activity of the liver.

The fundamental architecture of the bile canalicular system in mammals is that of a continuous, never-ending, polygonal meshwork. Intercellular branches, ending blindly—characteristic of the amphibian liver—are apparently of such infrequent occurrence in the normal mammal as to be insignificant.

The current concept of hepatic laminae in mammals, typically one hepatic cell in thickness, has been confirmed.

In the mammal there occurs with great frequency a more or less continuous, superficial or subcapsular lamina of hepatic cells, similar to the "limiting laminae" surrounding the portal spaces.

The normal mechanism of biliary excretion in the mammal, as in the frog, may be grossly distorted under conditions of anesthesia and anoxia.

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FIGURES 2–7

Explanation of Figures 2–7

Figures 2–7 are reproduced from the article by Hirt, Ansorge and Markstahler (2) by special permission, under License #JA-1546, dated March 5, 1952, issued by the Office of Alien Property, Department of Justice, Washington 25, D. C. The copyright, originally held by Verlag von Julius Springer, Berlin, Germany, is now vested in the U. S. Attorney General.

The original drawings for these figures were made by the distinguished German artist, August Vierling. From our experience with the fluorescent images obtained, the drawings are of such excellence that we could not hope to improve upon them. It should be emphasized, however, that Vierling's drawing of the normal architecture of the bile canaliculi in the rat (Fig. 2) does not properly illustrate the continuity of the pattern which we have regularly observed in mammals. The drawings were published by Hirt *et al.* in black and white, at a uniform magnification of 600×. As here reproduced, they have been modified by the addition of color to emphasize the distribution of fluorescein in the bile canalicular system and in the vacuoles within the hepatic cells. The task of coloring the figures has been accomplished with painstaking care by Leon Schlossberg, to whom we are deeply indebted.

Our Figure 2 is Hirt *et al.*'s Abb. 13, which bears the following legend: "Leber *Ratte.* Vergr. 600fach. Fluorescein. Völlige Ausfärbung der Gallencapillaren." Our Figure 3 is their Abb. 2, with this legend: "Leber *Frosch.* Vergr. 600fach. Fluoresceinausscheidung. Das Gallencapillarnetz ist mit den intercellulären Sekretcapillaren vollständig dargestellt. An der rechten Seite mehrere ampullenartige Erweiterungen der Gallencapillaren." Our Figure 4 is their Abb. 14, with this legend: "Leber *Ratte.* Vergr. 600fach. Von links nach rechts die fortschreitende Ausscheidung des Farbstoffes in einem Bilde. Links teilweise Dehnung der Gallencapillaren (maximale Füllung). In der Mitte beginnende Tröpfchenbildung. Rechts sehr starke Tröpfchenbildung." Our Figure 5 is their Abb. 3, with this legend: "Leber *Frosch.* Vergr. 600fach. Fortschreitende Fluoresceinausscheidung. Die zeitlich nacheinander folgenden Stadien der Tröpfchenbildung sind von links nach rechts in einem Bild dargestellt. Beachte die zahlreichen 'gestielten Tröpfchen'." Our Figure 6 is their Abb. 15, with this legend: "Leber *Ratte.* Vergr. 600fach. Fluorescein. Von oben nach unten Fortsetzung des Ausscheidungsvorganges. Zusammenfluss der kleinen Fluoresceintröpfchen zu grösseren Tropfen. Abbröckeln der Gallencapillaren. Die grossen Tropfen fliessen allmählich in die Gallencapillaren ab." Our Figure 7 is their Abb. 4, with this legend: "Leber *Frosch.* Vergr. 600fach. Fluoresceinausscheidung. Abbröckeln der Gallencapillaren. Zusammenfluss der kleineren Tröpfchen. Bei + ein Fluoresceinsee." In our Figure 7 this "+" has been deleted; the "Fluoresceinsee" so indicated in the original is the large, flask-shaped area in the lower right-hand corner of the figure.

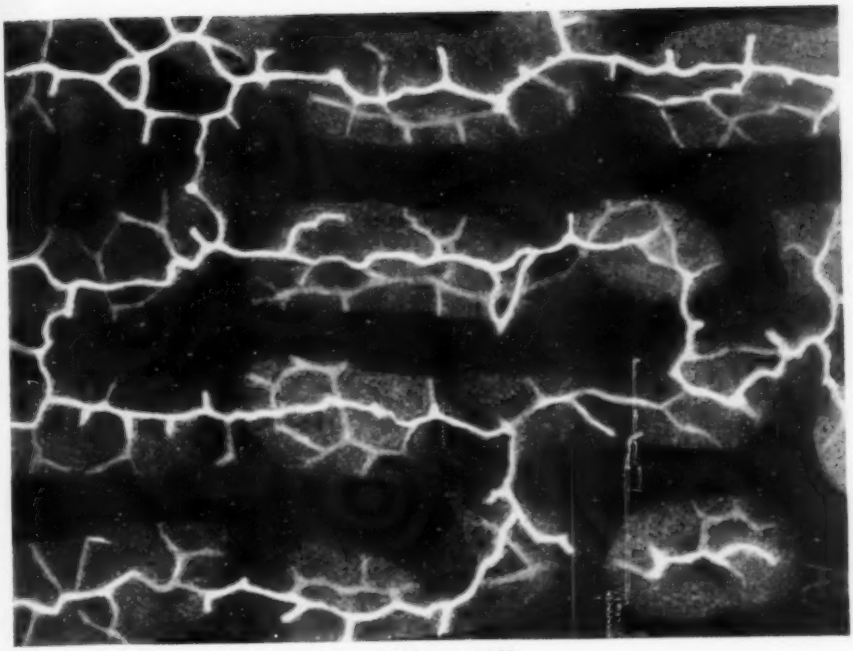

FIG. 2. Rat. ×1,000

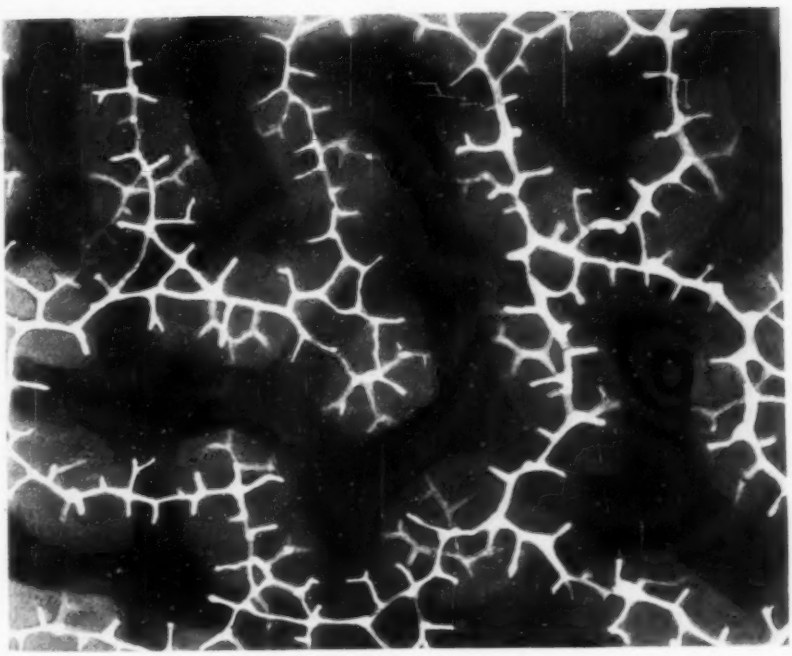

FIG. 3. Frog. ×700

Complete delineation of the bile canalicular system by fluorescein, at or near the peak of excretion. *Modified from Hirt, Ansorge and Markstahler (2).*

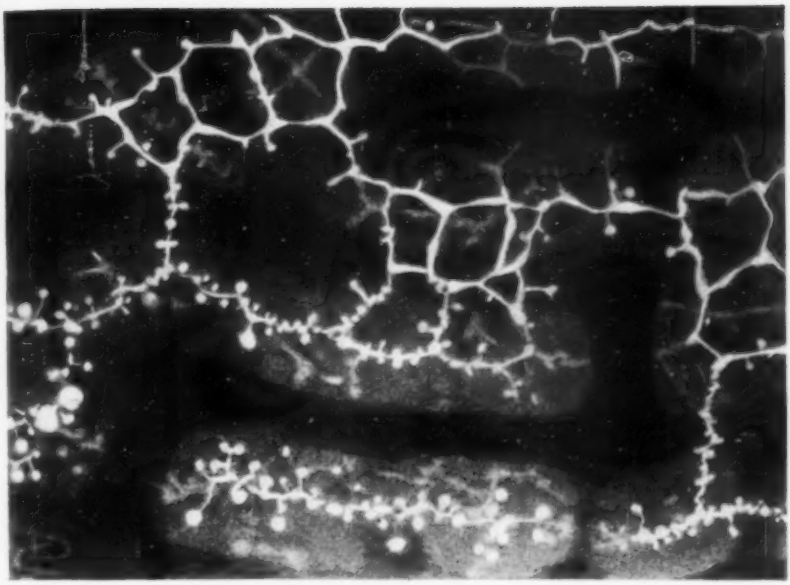

FIG. 4. Rat. ×1,000

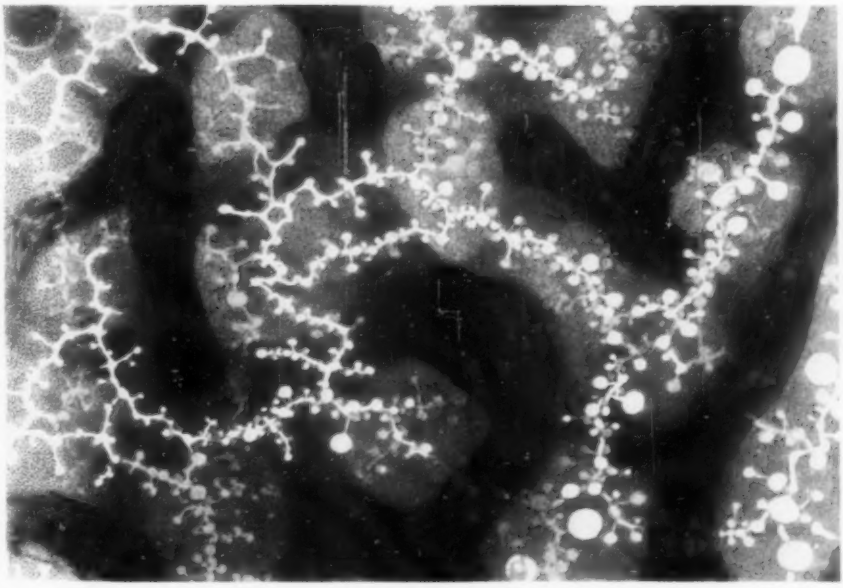

FIG. 5. Frog. ×700

"Sprouting" of fluorescein-containing vacuoles from bile canaliculi and intracellular vacuole formation, shown as a progressive picture, from above downward in Fig. 4, from left to right in Fig. 5. *Modified from Hirt, Ansorge and Markstahler (2).*

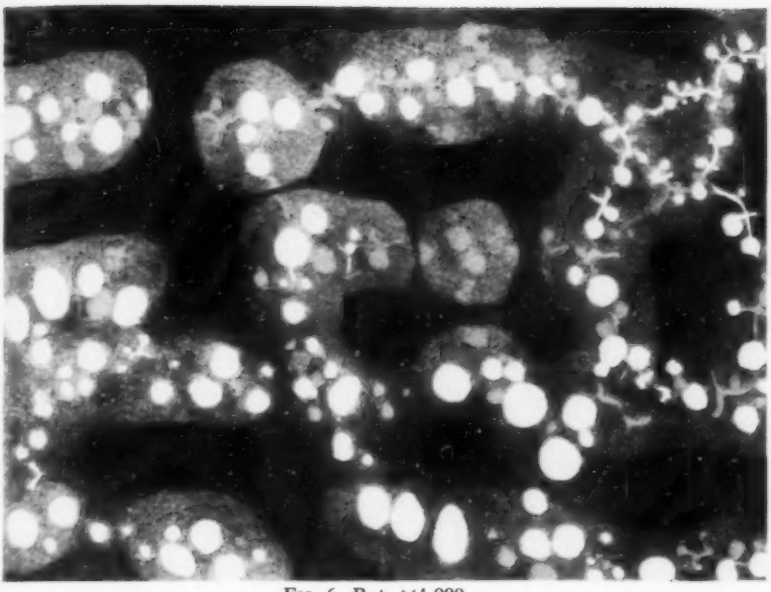

FIG. 6. Rat. ×1,000

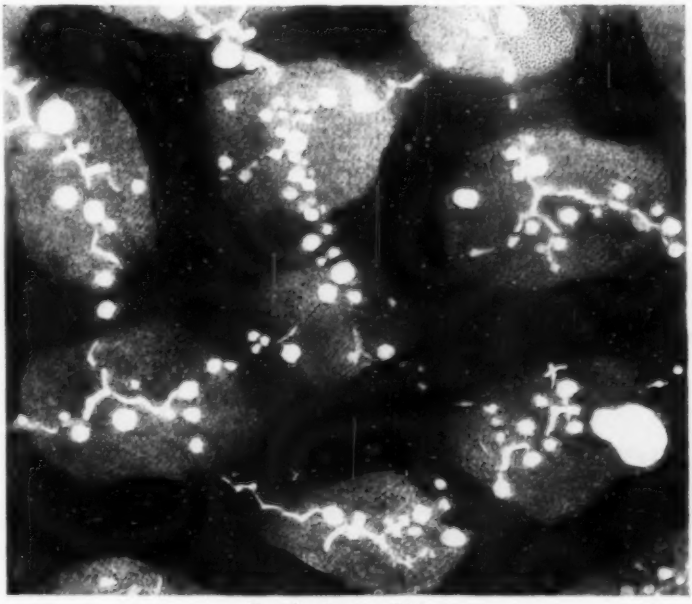

FIG. 7. Frog. ×700

Intracellular vacuole formation and discontinuity of bile canaliculi. *Modified from Hirt, Ansorge and Markstahler (2).*

THE FAILURE OF HISTAMINE TO REPRODUCE THE ARTHUS PHENOMENON

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The substance responsible for the phenomena of anaphylactic hypersensitivity has been the subject of much speculation and study. The view that has received the greatest favor is that the active substance is histamine. Following the studies of Dale and Laidlaw (1) and Dale (2) (3), which demonstrated that a condition closely similar to anaphylactic shock can be produced by the injection of a salt of histamine, many investigators have sought to test the hypothesis that the phenomena of anaphylactic hypersensitivity are due to the liberation of histamine as a result of the interaction of antigen and antibody in the tissues. There is an extensive literature on this subject, which it is not the purpose of this paper to discuss for there are numerous summaries of the pertinent evidence for and against this hypothesis (4) (5). It will suffice to say that while there is a widespread belief that the substance which produces the anaphylactic effects is histamine, not all investigators are convinced of this; and it is fair to say that, while some of the phenomena of anaphylaxis can be reproduced by histamine, it is certainly not yet established that histamine is the substance responsible for anaphylactic reactions.

Considerable weight has been placed upon the fact that, in addition to producing a state resembling anaphylactic shock when injected intravenously, the intracutaneous injection of salts of histamine produces an immediate and evanescent local wheal-and-erythema reaction which resembles the local reaction produced by the intracutaneous injection of the specific antigen into the anaphylactically sensitized body. Now it is well known that if the degree of anaphylactic sensitivity is increased by stimulating the production of increasingly large amounts of antibody, the local reaction produced by the injection of a given amount of antigen becomes increasingly larger and more severe until, at length, instead of a small, evanescent wheal-and-erythema, a large lesion characterized by massive edema, marked erythema, hemorrhage and necrosis results. This is the familiar "Arthus phenomenon." Keeping the intracutaneous test dose of antigen constant, one can, by increasing the level of circulating antibody, produce all degrees of severity of lesion ranging from the small, evanescent wheal-and-erythema to a huge hemorrhagic, inflammatory-necrotic lesion. If the liberation of histamine as a result of antigen-antibody interaction is the cause of the evanescent wheal-and-erythema anaphylactic

[1] Holder of Commonwealth Fund Travel Grant.

reaction, one may reasonably expect that the increasing degrees of severity of local reaction, up to and including the hemorrhagic-necrotic Arthus phenomenon, are caused by the liberation of increasingly large amounts of histamine; and if the wheal-and-erythema produced by the intracutaneous injection of a small amount of histamine is really the pathogenetic analogue of the anaphylactic wheal-and-erythema, the intracutaneous injection of a large amount of histamine should produce a replica of the hemorrhagic-necrotic Arthus phenomenon. The present study represents an attempt to produce the Arthus phenomenon by the injection of large amounts of histamine.

EXPERIMENTS

Male guinea pigs, weighing about 300 grams, and male albino rabbits, about 2,000 grams in weight, were used. The salt of histamine used was the diphosphate, and all solutions were freshly prepared immediately before each experiment, using sterile saline as the solvent.

Guinea pigs. In order to determine the minimal lethal dose of the histamine, solutions of increasing strength were injected intravenously via the jugular vein, and it was found that the smallest dose that would produce fatal shock, causing death within three to five minutes in three consecutive guinea pigs, was 0.7 mg. per kilogram of body weight. Having determined the lethal dose, high concentrations of freshly prepared solutions were injected into the shaved skin of the flank of different guinea pigs. The sites of injection were carefully observed and the resulting lesions charted.

One guinea pig (No. 83) was given intracutaneously 0.1 cc. of saline containing 10 mgm. of histamine diphosphate. This was 47 times the calculated minimal lethal intravenous dose for a guinea pig of the weight of this test animal. The animal developed respiratory distress shortly after the injection and died thirty-five minutes after the injection. Clearly, enough histamine had been absorbed to cause death, which occurred before significant alteration in the appearance of the site of injection had taken place.

Each of four guinea pigs (nos. 56, 60, 62 and 82) was given an intracutaneous injection of 5 mgm. of histamine diphosphate (about 23 times the calculated minimal lethal intravenous dose). All four developed respiratory distress, and two (nos. 60 and 82) died between 30 and 40 minutes after the injection. The other two recovered. The macroscopic character of the lesions in these animals is indicated in Table I.

Each of four guinea pigs (nos. 80, 81, 85, 92) was given 2.5 mgm. of histamine diphosphate intracutaneously (about 12 times the minimal lethal intravenous dose). These animals exhibited only slight respiratory distress and all survived. The macroscopic character of their lesions is indicated in Table II.

Only one guinea pig (no. 56) showed any macroscopic lesion at the end of 48 hours, and this was a trivial lesion in no way resembling an Arthus phenomenon (Table I). Microscopically, there was only slight edema, very slight infiltration of inflammatory cells and necrosis of a few muscle fibers beneath the corium, with proliferation of the adjacent perimyseal cells.

Each of the other guinea pigs that survived was given intravenously, at the end of 48 hours, the calculated minimal lethal dose, and all died in typical histamine shock, demonstrating that they were not refractory to histamine.

Rabbits. It was found that, for rabbits, the minimal lethal intravenous dose of histamine diphosphate per kilogram of body weight was larger than for guinea pigs. 7 mgm. per kilogram was the smallest intravenous dose that would kill rabbits in 3 to 5 minutes. This made

	G.P. 56	G.P. 62	G.P. 60	G.P. 82
10m.				
20m.				
30m			Died	Died
40m				
50m				
2hr.		—		
12hr.		—		
24hr.	O	—		
36hr.	O	—		
48hr.	O	—		

TABLE I. Skin reactions in guinea pigs injected intracutaneously with 23 times the intravenous minimal lethal dose. Dotted line shows actual size of papule.

⊙ Erythema.

O Ischemia.

— No abnormality visible.

	G.P. 80	G.P. 85	G.P. 92	G.P. 81
10m				
15m				
20m				
30m	—		—	
45m	—	—	—	—
60m.	—	—	—	—
2hr.	—	—	—	—
17hr.	—	—	—	—
24hr.	—	—	—	—
48hr.	—	—	—	—

TABLE II. Skin reactions in guinea pigs injected intracutaneously with 12 times the intravenous minimal lethal dose. See legend of Table I.

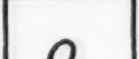
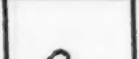
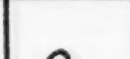
	R.1	R.2	R.3	R.4
10m.				
20m.				
30m				
40m				
50m				
60m				
2hr.				
17hr.				
36hr.				
48hr.				

TABLE III. Skin reactions in rabbits injected intracutaneously with about 2 times the intravenous minimal lethal dose. See legend of Table I.

it impossible to give as large an intracutaneous dose to rabbits, in proportion to their body weight, as was possible in the guinea pigs, for the solubility of the substance did not permit the preparation of solutions having more than between 2 and 3 times the minimal lethal intravenous dose for rabbits. The doses injected, however, represented the local deposition of truly massive amounts of histamine.

Two rabbits were injected intracutaneously with 20 mgm. of histamine diphosphate in 0.1 cc. of saline, and two were given 30 mgm. The macroscopic lesions are shown in Table III. Microscopically, the skin at the site of injection at the end of 48 hours showed a very slight edema and very slight infiltration with polymorphonuclear and mononuclear cells. There were several minute hemorrhages in two of the lesions. All showed a few necrotic muscle fibers with perimysial proliferation in the muscle layer immediately beneath the corium. In three rabbits (nos. 1, 2 and 4) there were alterations of several of the small arteries in the corium, consisting of focal hyalinization and slight leucocytic infiltration of the media and a few macrophages and polymorphonuclears in the adventitia. In places there was hemorrhage into the necrotic wall. These vascular changes resembled those of the arteriolonecrosis associated with hypertension more than the inflammatory-necrotic arterial lesions of the Arthus phenomenon. No alterations were seen in the veins. In no place was there thrombosis or more than a tiny hemorrhage, nor was there any appreciable swelling or degeneration of collagen fibers such as is characteristically marked in the Arthus phenomenon. In sum, the trivial lesions produced by these very large doses of histamine diphosphate bore no macroscopic resemblance whatever to the markedly edematous, erythematous, hemorrhagic and necrotizing Arthus phenomenon, nor did they exhibit the microscopic characteristics of the Arthus phenomenon (necrosis, thrombosis and intense inflammation of vessels, extensive edema and purulent exudate, marked swelling and degeneration of collagen fibers).

Since histamine diphosphate, in the doses used in the present experiments, produced prolonged local vascular spasm, in order to determine whether focal medial necrosis of arterial walls, such as that mentioned above, could result from prolonged vascular spasm, one rabbit was given an intracutaneous injection of 0.1 cc. of 1–1,000 epinephrine on one flank, and 0.1 cc. of 1–100 epinephrine on the opposite flank. The sites of injection were removed at the end of 40 hours. Microscopically, both sites showed approximately the same slight degree of edema, cellular infiltration and arterial necrosis as was present in the rabbits injected with histamine diphosphate.

DISCUSSION

The results of these experiments show that it was not possible to reproduce the Arthus phenomenon by the intracutaneous injection of histamine diphosphate in amounts even far larger than any amount of histamine that might reasonably be expected to be liberated at a local site of antigen-antibody interaction. It may be stated that amounts of the diphosphate smaller than those used in the present experiments likewise fail to produce anything resembling an Arthus phenomenon.

Dragstedt (5), in his review of the rôle of histamine in anaphylaxis, discussing the failure of histamine to cause the incoagulability of the blood that occurs in anaphylactic shock has concluded: "The liberation of histamine can account for practically all of the other (anaphylactic) effects, and there is no well defined prominent symptom demanding an alternative explanation." The

present experiments demonstrate that the injection into the tissues of even a massive dose of histamine, in the form of the diphosphate, is unable to produce the characteristic tissue changes of one of the most familiar expressions of anaphylactic hypersensitivity—the Arthus phenomenon. Since the view that histamine is the substance responsible for the phenomena of anaphylaxis has been built largely upon the fact that many of the manifestations of anaphylaxis can be reproduced by the injection of the hydrochloride or diphosphate of histamine, the present failure to produce the Arthus phenomenon by the injection of even very large amounts of histamine diphosphate requires consideration in relation to the hypothesis that the phenomena of anaphylactic hypersensitivity are due to the liberation of histamine into the tissues at the site of the antigen-antibody reaction.

CONCLUSION

In view of the widespread belief that histamine is the substance responsible for the phenomena of anaphylaxis, large amounts of histamine diphosphate were injected into the skin of rabbits and guinea pigs in the attempt to reproduce one of the characteristic anaphylactic reactions—the Arthus phenomenon. Nothing resembling an Arthus phenomenon resulted.

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MEETING OF THE JOHNS HOPKINS MEDICAL SOCIETY

MARCH 10, 1952

Dr. Grafflin: Before we get under way, I would like to state that the next meeting will not be held on the second Monday of the month but, instead, will be held on the third Monday, because of Easter. As in the past, this meeting will be devoted entirely to medical student research under the auspices of the Alpha Omega Alpha Fraternity. This will be on Monday, April 21st.

The first paper this evening is by Dr. Robert Austrian.

THE RELATION OF COLONIAL MORPHOLOGY AND ANTIGENIC STRUCTURE TO VIRULENCE IN PNEUMOCOCCUS

Although attempts to relate certain morphologic properties of bacteria to their ability to produce disease have yielded many useful data, the results of such studies have not always been consistent when a variety of bacterial species has been examined. There are notable exceptions to the association of bacterial virulence with smooth colonial forms and of avirulence with rough colonial variants. The isolation of rough encapsulated strains of pneumococcus and an examination of their properties has permitted a reevaluation of the relation of colonial morphology and of antigenic structure to virulence in this species.

Four colonial variants of pneumococcus may now be recognized: smooth encapsulated, smooth unencapsulated, rough encapsulated and rough unencapsulated. Smooth forms differ from rough forms in their mode of cellular separation after division, the former separating characteristically into single cells or pairs whereas the latter remain attached after division forming chains of great length. The basis for this difference in the growth of smooth and rough variants is at present unknown. Encapsulated pneumococci differ from unencapsulated variants of the same species by virtue of their ability to produce a type-specific surface polysaccharide which gives rise to the mucoid appearance of colonies of encapsulated cells. Studies employing selective cultural techniques suggest strongly that the factors controlling encapsulation and mode of cell separation after division may vary independently of one another. This impression has been confirmed through the use of pneumococcal transforming reactions. When smooth unencapsulated pneumococci are transformed in the presence of the desoxyribonucleic acids (DNA) of a rough encapsulated strain, rough unencapsulated and smooth encapsulated variants can be recovered. Similarly, when rough unencapsulated strains are transformed in the presence of DNA from a smooth encapsulated variant, rough encapsulated and smooth unencapsulated strains are observed. These results establish conclusively the independent heritability of the factors controlling encapsulation and mode of cell separation after division.

Smooth and rough unencapsulated pneumococci possess both species-specific C carbohydrate and type-specific M protein. They are phagocytized, however, by human leukocytes in the absence of added antiserum and are avirulent for mice. Smooth and rough encapsulated variants possess, in addition to the two antigens mentioned previously, type-specific capsular polysaccharide. Strains of these two variants are not phagocytized in glass systems in the absence of homologous capsular antiserum and are virulent for mice.

The observations reported confirm the earlier findings of many workers concerning the importance of encapsulation to virulence in pneumococcus. They reveal also that, in pneumococcus, the presence of the capsular antigen may endow this organism with the ability to infect irrespective of its association with cells multiplying in the pattern of either smooth or

rough variants. The fact that a surface antigen of major importance to virulence may vary independently of and be associated with either smooth or rough colonial forms may explain certain of the discrepancies which have arisen previously in attempts to correlate the colonial morphology and virulence of bacteria.

Dr. Grafflin: Dr. Harvey, would you care to discuss this paper?

Dr. Harvey: I have nothing to say in discussion except to compliment Dr. Austrian on his presentation of a quite complex subject. I am quite certain that Dr. Bang and others are better able to discuss the technical details.

Dr. Grafflin: Dr. Bang.

Dr. Bang: I can just add to Dr. Harvey's compliment of Dr. Austrian's presentation. There is one minor question I would like to ask and that is whether the addition of antibody to a smooth virulent form allows for any mimicking of this rough virulent form?

Dr. Austrian: There are a number of environmental factors which may lead genotypically smooth organisms to assume the appearance of rough variants. In the late nineteenth century, several bacteriologists showed that pneumococci, cultivated in the presence of homologous immune serum, grew in long chains. More recently it has been shown by Okamato and Shako that a variety of quaternary ammonium compounds, notably choline, will cause genotypically smooth pneumococci to grow in long chains in liquid or on solid media. In our laboratory, we have obtained similar alterations in the growth pattern of smooth pneumococcal variants by altering the ionic composition of the culture medium. These changes, however, are not genetic and persist only in the special environment.

At present we have very little knowledge regarding the cytochemical differences between rough and smooth cells. It is possible that smooth variants possess an enzyme activated by divalent cations which leads to cellular separation after division and that this hypothetical enzyme is lacking in rough variants. Alternatively, both variants may possess the hypothetical enzyme but an inhibitor of its activity may be present in rough variants. The problem has been explored so little that, at present, one can only speculate upon the various factors which may influence cellular separation after division.

Dr. Grafflin: Is there any other discussion from the floor? If not, we will go on to the second paper, which is by Dr. P. K. Duraiswami.

EXPERIMENTAL INDUCTION OF CONGENITAL DEFECTS

In recent years it has been shown by various workers (such as Hale, Warkany, Byerly, Romanoff, Couch, Lyons, Bagg, Little, Landauer, Duraiswami, Karnofsky, Ancel, Lallemand, Ackermann, Taylor and others), that certain types of congenital defects can be induced in the offspring not only by maternal nutritional deficiencies and Roentgen irradiation but also, at least in some animals, by the deliberate introduction of certain substances such as insulin, cortisone, sulfonamide compounds, thallium salts and 3-acetylpyridine into the environment of the developing embryo. Various congenital skeletal abnormalities broadly resembling those which occur in the human have been induced in developing chickens by injecting different doses of insulin into the yolk of fertilized eggs, the type of deformity depending on the time of injection after the beginning of incubation of the eggs and the dose of insulin. Abnormalities thus induced included partial or complete suppression

of development of one or more vertebrae, scoliosis, spina bifida, adduction deformity of the digits ("club feet"), congenital dislocation of the hips and knees, "arthrogryposis multiplex congenita," pseudarthrosis of the tibia and "tibial kyphosis." A generalized developmental disturbance of bone resembling in certain respects "osteogenesis imperfecta" in the human had been induced with high doses of insulin. Some of the above-mentioned abnormalities were illustrated by lantern slides and a cine film, showing the deformities in live chickens of various ages. Insulin-induced abnormalities could be prevented to a remarkable extent by injection of nicotinamide *plus* riboflavin into the yolk at the same time as insulin.

Some of the congenital defects induced by cortisone (Merck's cortone acetate ®), "soluseptasine" (a May & Baker sulfonamide compound), thallium nitrate and 3-acetyl-pyridine were also illustrated by lantern slides. These included limb and beak deformities caused by "soluseptasine" and thallium nitrate; "myodystrophy" induced by 3-acetyl-pyridine; and stunting of growth, ectopia viscerae (exomphalos with or without ectopia cordis), blister-like blebs, tight amniotic sacs and amniotic bands, produced by cortisone. It was also shown that if cortisone was injected into the yolk *in addition to* each of the following substances: thallium nitrate; 3-acetyl-pyridine; "soluseptasine"; and insulin, the afore-mentioned abnormalities induced by cortisone viz., stunting of growth, ectopia viscera and etc., appear *in conjunction with* the appropriate defects caused by each of the other substances enumerated, thus indicating the specificity of action of each teratogenic agent.

The experiments involving the injection of cortisone, either by itself or in combination with insulin, thallium nitrate, 3-acetyl-pyridine and 'soluseptasine' have been conducted with the aid of the Bowles Orthopedic Research Fund in the Children's Hospital School, Baltimore, while the other experiments had been carried out in India and the United Kingdom.

Dr. Grafflin: Professor Corner, would you care to comment on this?

Dr. Corner: Dr. Duraiswami has presented here a topic that can be discussed at any length by embryologists and pathologists and I am sure there are many here who can discuss the contribution better than I. There are, however, three major points calling for comment by one who represents embryology of another sort.

In the first place, Dr. Duraiswami's experience is closely in line with that of other experimental embryologists the world over who have been emphasizing and re-emphasizing that specific embryonic deformities, defects, and retardations can be produced by carefully timed and graded toxic and traumatic agents. At present, the wave of thought in regard to the nature of congenital defects in mammals has been influenced by these experiments on other vertebrates, and people are thinking more and more, and experimenting more and more, in this way; that is to say, influencing development by environmental changes. At the same time, we must not forget there are hereditary or congenital defects in man and the next thing to do is to find out what proportion of the cases are produced by genetic causes and what proportion by environmental disturbances. For example, the condition of "rumplessness" of the chick, which has been mentioned here, has been demonstrated to occur as a hereditary defect. The same lesion of the lower spine has also been produced by C. H. Danforth by changing the temperature during incubation and I understand that Landauer too, has produced it by injection of insulin into the egg. It has also been observed to occur sporadically without any discernible cause. This merely emphasizes the fact to which Dr. Duraiswami referred, that the noxious agent, whether a chemically defective environment or a mechanical injury, may hit the same sensitive growing region and produce identical results.

Dr. Duraiswami has gone farther than many previous investigators in drawing comparisons between experimental animals and human defects. He is interested in the pathology, as seen through the eyes of a medical pathologist, and wins our interest at once through that way of studying those specimens. It seems a good thing to have experimental embryology done in fish, amphibia and birds by medical men, in medical circles, if only to teach us that man is one of many animals subject to similar pathological disturbances.

The third point that I have on my mind with regard to this extremely interesting presentation is the question of apparent production of defects recurring in the second generation after treatment of the first generation only. This is a highly debatable question which has caused endless difficulty in the past. Numerous reports of changes induced by environment being transmitted to the next generation, have failed to be substantiated by subsequent critical experiment. Dr. Duraiswami is aware that what he has said on this point might well be received with a high order of skepticism, and he has spoken cautiously. If it is to be proved, as of course it may be, it will require a large statistical series, done with birds of known genetic stock, and carefully guarded experimental control. I hope, for the sake of scientific interest, that other workers will follow up this research and see what can be done to confirm this particular point.

Dr. Grafflin: Professor Johnson.

Dr. Johnson: I would like to say that this demonstration that Dr. Duraiswami has given here tonight and last year in Chicago, was so fascinating to me in the apparent similarity between these induced deformities and the congenitally deformed children we have had to treat for years without knowing more than the fact that it was vaguely hereditary, that I just couldn't help wanting to have him come here and work close to me but in an entirely independent fashion, so that he could do exactly what he wanted with the material and carry out any ideas that he had. I am fortunate in having a research fund available which is not under any other restrictions than the proper use that I choose to designate it for. This seemed to be a golden opportunity to see what could be carried out here on the basis of the work that he had done so carefully in Liverpool for his Ph.D. thesis.

As I have watched the production of the subsequent deformities that he has produced here in the laboratory of the Children's Hospital School, it is perfectly astounding to see these chicks appear with almost any of the deformities he has depicted in this demonstration —really it is almost a shock. He can tell us what to expect, and when it will appear, according to the time of dosage, combination of drugs, etc. All this is fascinating, but I think it has a deeper implication. It appears to me to be this way.

We have a method to arrest, or damage, certain biological changes taking place, particularly in the cartilage of the developing limb buds at a given time and, in that way, may be able, by vital stains, or by other biochemical methods to determine just what happens in the cell masses at that time, and perhaps learn a little bit more about the intimate biochemistry of the collagen system.

We have here the collagen materials affected with certain of the injections, such as insulin. It predominates in the skeletal structure in the prebone cartilage. In others cases there is an effect on the muscular system. This widespread action is certainly indicated in a case we had at the clinical pathological conference last week—a very interesting osteogenesis imperfecta in a human infant with notable changes not only in the spinal column and skeleton generally but in the eye capsule and subcutaneous collagen tissue. So we hope to have here a very potent opportunity to study the formation or malformation, or arrest, of a collagen tissue at a critically labile stage and I think that this is positively the greatest single

thing I can foresee coming out of this basic piece of work. I hope a great many people will grab hold of the method. It is work, not for one man, but for a great many men approaching it from different angles and I hope it will be utilized widely in studies to give us a better understanding of the whole problem of collagen formation.

Dr. Grafflin: The time is getting away from us, and out of fairness to the third speaker we will now have the last paper by Drs. Theda R. Hopkins, Thomas B. Connor and John Eager Howard. The paper will be presented by Dr. Howard.

STUDIES ON THE TRANSPORT OF CALCIUM AND PHOSPHORUS IN THE SERUM WITH SOME THOUGHTS ON THE FUNCTION OF THE PARATHYROID GLANDS*

Using the technique of Lavietes, in which an ultrafiltrate of serum can be obtained anaerobically and with controlled pH, it was found that at pH 7.4 the calcium concentration of ultrafiltrate is approximately 70% that of the unfiltered serum. Phosphorus concentration of the ultrafiltrate is almost identical with that of the whole serum. To pooled normal serum when calcium is added up to a total concentration of about 20 mg.%, the calcium concentration of the ultrafiltrate continues to be 70% of that of the unfiltered serum, and the phosphorus concentration is the same on the two sides of the membrane. However, when concentrations of calcium greater than 20 mg.% are reached, the ultrafiltrate shows a reduced concentration of phosphorus and usually calcium, as though some sort of non-filterable calcium-phosphorus-protein complex had been formed.

When, over a 4-hour period, calcium is given intravenously as gluconate or glucoheptonate in normal saline, so as to raise serum calcium from 10 to 14 or 15 mg.%, there is found a rise in serum phosphorus associated with a fall in urine phosphorus. The inference is that function of parathyroid glands has been reduced by the hypercalcemia. Calcium so administered to six patients with later proven parathyroid adenomas, resulted in lesser rise in serum phosphorus and a *rise* in urinary phosphorus.

From these observations there are developed some hypotheses as to how the parathyroid hormone may act as a major factor in controlling the calcium concentration of the serum.

Dr. Grafflin: Dr. Harvey, would you care to comment on this paper?

Dr. Harvey: How is the calcium in the filtrate influenced when the proteins in the serum are abnormal in some such condition as myeloma?

Dr. Howard: We have thus far carried out very few ultrafiltration tests on sera with abnormal protein content. We should be glad to have sera in such cases either with or without hypercalcemia. One patient's serum was examined which contained 12 grams of protein, 10 grams being globulin, and also was mildly hypercalcemic with 12 mgm. per cent calcium. The ultrafilterable calcium in this instance proved to be 90 per cent; that is, the calcium in the ultrafiltrate was 10.5 mgm. per cent, a very high figure. This patient suffered from multiple myeloma and had widespread bone disease by x-ray. Our impression was that there was very active osteolytic action pouring out much lime salt into the blood stream. Evidently the particular globulin in this case had very little capacity to bind calcium.

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* The studies described in this paper were carried out under a contract between The Johns Hopkins University and the Atomic Energy Commission.

Dr. Harvey: When the ultrafiltrate calcium is elevated or suppressed, presumably this would affect neuromuscular function. What do you guess this change would be in a patient with hypercalcemia and abnormal protein and how might it affect the parathyroid function?

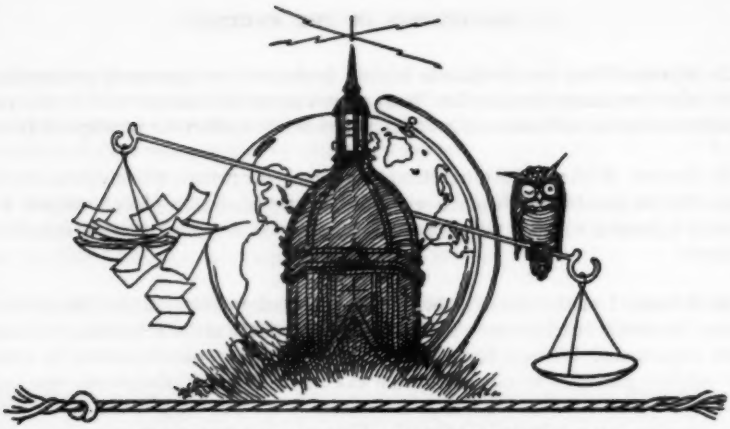
Dr. Howard: With the high ultrafilterable calcium in our patient with myeloma, we should guess that the parathyroid function would be suppressed. Serum phosphorus was a little elevated in keeping with this view. Neuromuscular mechanisms were certainly not obviously abnormal.

Dr. Wilkins: I was interested in the case of hypoparathyroidism that Dr. Howard showed because his results fitted in very well with the concept of the action of parathyroid hormone which he presented. We have just studied an infant with hypoparathyroidism to whom we gave calcium gluconate to raise the serum calcium. The serum phosphorus rose and the urinary phosphorus fell the same as in the normal. That may suggest this infant does not have complete hypoparathyroid deficiency. Clinically, however, we have no evidence that this is not a full and complete case.

Dr Grafflin: Is there any other discussion?

Before closing, I would like to thank our speakers, who have given notable presentations of three quite diverse papers and very interesting ones.

The meeting is adjourned.



BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of the Bulletin)

The Pathogenesis of Tuberculosis, 2nd Edition. By ARNOLD R. RICH. 1018 pp., $15.00. *Charles C. Thomas, Springfield, Illinois.*

It is the fashion just now in medicine to be rather casual about the infectious diseases, even those which like tuberculosis continue year after year to take their toll among both young and old. It is estimated that there are 500,000 persons with active tuberculosis in the United States, with 100,000 new cases occurring each year. In large areas of the world the death rate exceeds 100 per 100,000 population as compared with approximately 22 per 100,000 in this country.

The publication of a scholarly and authoritative monograph on the subject by an outstanding investigator in the field is therefore an event of significance. The Second Edition of *The Pathogenesis of Tuberculosis* by Arnold R. Rich incorporates the recent advances in our knowledge of this disease, and more importantly reflects a judgment tempered by seven additional years of study and experience.

This is really two books in one. As its title indicates it is primarily a treatise on tuberculosis, in which the great accumulations of fact and near-fact in the realms of bacteriology, immunology, pathology, clinical observations, epidemiology, and genetics are assembled, integrated, and critically examined. The result is a book which conveys a degree of understanding of the exceedingly complex interplay of factors inherent in the host-parasite relationship in this disease; a relationship which may now be modified for good or ill by such modern developments as antibiotics, artificial immunization, and the adreno-corticotrophic hormones.

The student of tuberculosis will find here almost all that is known of the fundamental processes of the disease; the clinician and public health officer will gain a deeper insight into the forces that promote illness or recovery, community spread or limitation.

But quite beyond this one disease, Arnold Rich has written a fine book on the fundamental biology of infectious processes. That tuberculosis happens to be the model, illuminates rather

than limits the value of the profound exploration of this important subject. One need only read such chapters as those devoted to a discussion of the relation of the chemical constituents of the tubercle bacillus to pathogenesis, the nature and effects of virulence, the mechanism of native resistance, acquired resistance, and hypersensitivity, and the factors responsible for the characteristics of tuberculous lesions and symptoms, to realize that here too is a book that physicians and biologists whatever their special interest may be, could study with profit.

Amid the welter of medical books produced by the "multiple-author" process it is gratifying—and rewarding—to see an important aspect of medicine developed with some consideration for the literary virtues of unity, coherence, and emphasis; and an exciting experience to share the workings of a critical and creative mind.

The book has 105 informative illustrations and a good index. The First Edition was translated into the Spanish; it is to be hoped that this edition will be made even more widely available.

THOMAS B. TURNER

Adrenal Cortex. Transactions of the Second Conference, November 16–17, 1950, New York. Edited by ELAINE P. RALLI. 209 pp., $3.00. *Josiah Macy, Jr. Foundation, New York.*

This book contains informal presentations and highly informal discussion on various aspects of adrenal cortical physiology by acknowledged experts in the field. This conference covers the present status of the chemistry of ACTH, theories on the regulation of ACTH output by the pituitary, some experimental evidence on the mechanism of action of adrenal steroids against hypersensitivity and the relationship of the cortical steroids to "spreading factors".

The authoritative prestige of the participants and the informality of the discussion make good reading for the workers active in the field of adrenal physiology. There is also much important clinical experience described which is highly valuable to clinicians dealing with Addison's disease, Cushing's disease and for those who must use ACTH or cortisone for the pharmacological action of these compounds.

J. E. H.

Connective Tissues. Transactions of the Second Conference, May 24–25, 1951. Edited by CHARLES RAGAN. 190 pp., $3.50. *Josiah Macy, Jr. Foundation, New York.*

This is another of the stimulating transactions of the Macy Foundation conferences, in particular, a report of the second conference on connective tissues. While it is in no way a comprehensive review of our present knowledge of connective tissues, it is an often exciting presentation by eminent investigators with informal discussions by a select group of basic and clinical scientists interested in the problems. The results of recent studies as well as suggested future investigations concerning the ground substance of connective tissues, the chemistry of elastic fibers, pain mechanisms associated with certain foci in connective tissue sites, repair processes and the regression of scar tissue are discussed. These topics are considered by the speakers in light of their own particular researches or special techniques. The broad implications of the subject, "repair processes" assumes the minute perspective of the formation of extracellular fibers by cells as seen under the electron microscope and the conclusions drawn here do not seem to be entirely warranted. "Regression of scar tissue" is concerned solely with the disappearance of collagen from experimental cirrhosis. Round table discussions broaden these views. The topics "ground substance" and "elastic fibers" are brilliantly presented and Dr. Travell's discourse on pain, new to this reviewer, must have broad clinical implications.

In all, a large number of very recent and exciting observations dealing with the connective tissues are presented and discussed in the novel and stimulating style used by the editors of the many Macy Foundation Conference Transactions. The occasional distraction occurring with the interruption of the speaker by questions from the discussers does not significantly detract from the excellence of this edition.

M. B.

Annual Report on Stress. By HANS SELYE. 511 pp., $10.00. *Acta Inc., Montreal, Canada.*

Ninety four years ago, Claude Bernard described the role of the glands which we now call endocrine in the maintenance of the ***milieu interne***. Seventy-four more years passed before W. B. Cannon's definitive presentation of his life-long study of homeostasis. In a period dominated by bacterial theories of disease these concepts have done little to form our ideas of pathological processes. However, during the last ten years, Selye has advanced a development of the earlier hypotheses, in which he seeks to show that the physiological mechanisms concerned in homeostasis may become unbalanced and cause what he has called "diseases of adaptation". It has long been known that removal of the adrenal cortex in most mammals causes major disturbances of the internal environment which are incompatible with life. The normal secretion of the adrenal is known to be controlled humorally by the anterior pituitary, which in its turn is thought to be under the control of the central nervous system, probably through the hypothalamus. It is also common ground among physiologists that a great variety of external stimuli may cause reflex stimulation of the adrenal cortex, resulting in a number of metabolic and other effects—gluconeogenesis at the expense of body protein, involution of the thymus and lymphatic tissues, and eosinopenia, for example. Selye regards this adrenal secretion as the principal homeostatic mechanism by which the organsim reacts to "stress", distinguishing it sharply from the specific processes which are defensive against only one stressor agent, such as serological reactions to antigens, muscular hypertrophy after exercise and so on. His discovery that the administration of large doses of the substance DCA, which resembles one type of adrenal steroid in its action, could produce changes resembling a variety of disease processes in specially sensitized experimental animals, led him to suggest that such diseases themselves might be the result of prolonged exposure to stress. His conclusions have not been accepted without considerable criticism; DCA is probably not secreted by the adrenal, the doses employed were very large, and the resemblance between the experimental lesions and human diseases is superficial. The lesions in rat kidneys for example can only be produced after unilateral nephrectomy and the administration of salt, and Follis has pointed out that they are histologically identical with those which occur in potassium deficiency, which is probably produced by the heroic treatment the rats are given. In any case, as Goldblatt has pointed out, the rat is subject to degenerative lesions of this type even under normal conditions, especially as it grows older. Some of these objections are dealt with vigorously, if not altogether convincingly, in the introduction to present volume.

In 1949, Hench demonstrated the therapeutic effect of an adrenal cortical steroid in one of the conditions which Selye had classified as a disease of adaptation. Subsequently it has been shown that cortisone has a palliative action in a great variety of diseases, some of which fall within Selye's classification. These apparently empirical discoveries demand an explanation, and Selye claims that his hypothesis supplies it, although he makes it clear in the present book that his work was not the theoretical basis for Hench's clinical trial. The point which Hench and many others have found difficult to reconcile with the therapeutic effect of cortisone, is that Selye claims that the diseases are actually produced by increased adrenal secretion. Selye's answer is that they are produced by an excess of what he calls 'mineralo-

corticoids', and that the action of these is antagonistic to the cortisone type of substance, or 'gluco-corticoids' as he prefers to call them. Yet there is a growing body of evidence that ACTH causes the adrenal to secrete principally compound F, a substance almost identical in its effect with cortisone, and has very little effect on the secretion of salt retaining hormones. Analysis of blood from the adrenal vein of the intact animal has confirmed that this is also true in stress; in other words stress increases the secretion of the protective, rather than the damaging type of substance.

Some further chapters are devoted to a discussion of the wider theory of what the author has called the "General Adaptation Syndrome". The essence of this is the observation, as familiar perhaps to the laity as to the physiologist, that an animal's reaction to a noxious change in its environment may alter with the time of exposure. A load which causes some initial collapse may come to be borne with comparative ease after training, and yet may lead to exhaustion after prolonged exposure. There is little objection, if little novelty, to a generalised description of this process as the "alarm reaction", consisting of "shock" followed by "counter shock", a "stage of resistance" and a "stage of exhaustion". The prolix definitions by which these simple terms are fitted into Selye's complex and esoteric physiology are not so easily accepted, however. The stage of resistance, for example, is "the sum of all non-specific systemic phenomena elicited by prolonged exposure to stimuli to which the organism has acquired adaptation". This insistence upon non-specificity has already been referred to. Yet we are told that the resistence increases only to the particular agent to which the animal is adapted, the "crossed resistance" to other stressor agents being markedly diminished. It would seem more reasonable to explain this in terms of the specific responses which Selye rejects.

The term stress itself, it is claimed, is used in the sense "in which it has long been used in physics". However, it often appears to include concepts which the physicist would describe as load and as strain. One critic has expressed this succinctly as "Stress, in addition to being itself and the result of itself, also causes itself". It may have given the critic some satisfaction, after having his suggestion dismissed with contumely in chapter 1, to find it adopted at least in part, two chapters later when stress is distinguished from the stressor agent (load?). It would conflict less with common usage, as well as with physics, if the term were to be restricted to a description of the state of tension set up in the animal by the external load, and the physiological changes were to be described as strain.

One of the purposes of the present monograph, which the author freely admits, is the polemic one of interpreting the vast literature which has grown out of the study of adrenal physiology and the therapeutic use of adrenal substances, in the light of the author's personal hypothesis. At the same time he has undertaken the didactic task of providing an unbiased guide to this literature. The first of these aims has so determined the method of presentation of the material as to limit the success achieved in the second. It is difficult, for example, to follow the reasoning by which congenital adrenal hyperplasia, a condition which is known to originate in the first trimester of intra-uterine life, comes to be classified as an adrenal disease of adaptation. Nor is it easy to justify the invention of the neologism "hypo-mineralocorticoidism" for the variant of the same clinical condition in which there is an excessive loss of salt in the urine, but no evidence as to whether this loss is due to a lack of salt-retaining hormone or the secretion of some abnormal steroid. The frequency of such examples throughout the book must result in its being misleading to the clinician who is not familiar with this branch of medicine, and it certainly hinders its quick use as a work of reference. Even the index suffers from the inclusion of the neologisms and abbreviations of which the author is so fond, and the omission in many cases of the more customary names; it is difficult therefore to make the best use of the extensive bibliography of some three thousand recent

references. This is a difficult book to read, and while it will undoubtedly find a place in most medical libraries, it will make little appeal to the general reader, or the busy clinician seeking an interpretation of the therapeutic effects of adrenal steroids. Perhaps its most serious fault is the impression which it leaves with the reader that the facts are being tailored to fit the hypothesis.

G. C. K.

Studies in Medicine. A Volume of Papers in Honor of Robert Wood Keeton. By Various Authors. 396 pp., $8.50. *Charles C. Thomas, Springfield, Illinois.*

This commemorative volume has been compiled to honor Dr. Robert W. Keeton upon his retirement in August 1951 after eighteen years as head of the Department of Medicine at the University of Illinois College of Medicine. There is a six page biography of Dr. Keeton, and a complete bibliography of his one hundred and twenty papers, 1914–51. As pharmacologist, physiologist, and clinician, Dr. Keeton's range of interests focused on nutrition, obesity, diabetes, the response of the individual to environmental change, gastric functions, and convalescence. His life and teachings influenced a large number of students and associates and established his welcome by many scientific societies.

In this work there are thirty articles which have been contributed by fifty seven of Keeton's professional friends, mostly of the University of Illinois. This community of friends covers a diversity of subjects which are preponderantly on metabolism in accord with Dr. Keeton's own interests. In parts the volume is a technical manual, elsewhere it is a reference for the medical practitioner. For example, the table of contents includes such variegations as "The Control of Environmental Factors in Clinical Research;" "The Diagnosis of Pulmonic Stenosis;" "Psychiatric Implications of Dental Erosion;" "The Microdetermination of Lipids;" "Primary Tumors of the Renal Pelvis and Ureter;" "The Behavior of Eosinophils;" "Circulatory Failure as a Cause of Fever;" "Relationships in Cirrhosis;" "The Art of Using Lantern Slides". Ricketts and Goldner have written on allergy and immunity to insulin. Percival Bailey has contributed, "A Stereotaxic Instrument for Use on the Human Brain." Several chapters are devoted to studies of nutrition and obesity. Carroll Birch has written on clotting mechanisms, Warrent Cole on the role of nutrition in surgery, Arthur Colwell on management of diabetes in children, and W. O. Thompson on the clinical applications of ACTH and cortisone. These articles are of good quality and in general summarize the individual author's work in a specific field.

This panoramic volume is recommended to those who have known Dr. Keeton personally or professionally and to those who are interested in keeping abreast of studies in metabolism and nutrition by the Illinois group. The volume will of course mean most to Dr. Keeton himself who constitutes its *raison d'etre.*

G. W. D.

Current Therapy 1952. Edited by HOWARD F. CONN. 849 pp., $11.00. *W. B. Saunders Co., Philadelphia, Pa.*

This is the third issue of an annual compilation of methods of treatment written as individual original contributions by men who are authorities in their various fields. The editor has included a number of new contributions and has omitted some previous contributors. Each article has been reviewed and brought up to date so as to include methods of treatment which have been developed since the previous issue. By comparing the previous issues on a given topic one may find opinions by a number of authorities. This fact coupled with excellent coverage of new methods justifies the annual publication of this book. It should prove of more value to physicians who have a busy generalized practice than to those who are narrowly specialized.

R. E. M.

The Specialties in General Practice. Edited by RUSSELL L. CECIL. 818 pp., $14.50. *W. B. Saunders Co., Philadelphia, Pa.*

The authors of this book set out upon the Herculean task of presenting to the general practitioner the essentials of the various specialities as they most commonly affect him. These authors, distinguished men in their respective fields, have succeeded remarkably in bringing to the family doctor a wealth of practical experience in diagnosis and management. It is a book to which he may refer quickly without recourse to weightier specialist tomes and find out the most important practical data in the management of a case. Of necessity various methods are not discussed at great length but the treatments and opinions expressed are generally widely accepted. Emphasis is properly placed on whether or not the general practitioner, according to his experience should attempt to manage the case, what preliminary investigations he may carry out and what emergency treatment if any he should attempt if he is unable to obtain expert help. The information in the book is obviously culled from the authors' vast personal experience and contains much helpful advice on the management and comfort of patients during various therapeutic and diagnostic procedures. These are well exemplified in the opening of the first section on preoperative preparation of the minor surgical patient and the discussion of the proctological examination in the section on the Diseases of the Anus, Colon and Rectum.

The book is divided into fourteen sections which are as follows: Minor Surgery, Orthopedic Surgery, Fractures and Dislocations, Urology, Diseases of the Anus, Colon and Rectum, Gynecology, Obstetrics, Pediatrics, Ophthalmology, Diseases of the Nose and Throat, Diseases of the Larynx, Bronchi and Esophagus, Otology, Dermatology and Syphilology, Psychiatry. These are all surgical specialites with the exception of the last two. The inclusion of one or two more medical specialties such as neurology and gastroenterology might have been advantageous.

The first chapter on Minor Surgery is divided into preoperative preparation, analgesia and anesthesia, postoperative care, congenital anomalies, trauma, infections, neoplasms, miscellaneous conditions and routine procedures such as thoracentesis, venapuncture, etc. Some may not agree with cauterization of bites with fuming nitric acid, and the local application of penicillin ointments around furuncles after opening. The illustration F on page 38 appears to contradict the text on page 34 regarding the proper incision for acute paronychia. These are minor criticisms in what is by and large an excellent chapter.

Chapters two and three are devoted to Orthopedics and are divided into sections on orthopedic surgery, and dislocations and fractures respectively. These chapters are profusely illustrated. The painful back is adequately discussed and there is a fracture treatment guide for the general practitioner where the first aid treatment for fractures, complications of fractures, anaesthesia, reduction, immobilization and convalescence are tabulated. Thus the information may be obtained at a glance. The text amplifies this and properly emphasizes the dangers of treatment beyond the capabilities of the attending physician.

Dr. Jewett, of this Hospital, has contributed the section on Urology. He has approached his subject in a somewhat different manner from the other authors in that he elaborates upon genito-urinary symptomatology in his first chapters. Included in this section is an excellent description of the clinical examination of the genito-urinary patient.

The next chapter, Chapter 5, devoted to diseases of the anus, rectum and colon was written by Charles G. Child III. This is probably one of the best sections in the book. It is extremely practical. The approach to and the consideration of the comfort of the anorectal patient is emphasized. The general practioner's obligations as far as his investigations are concerned are specified and the proctological examination is discussed at length. The subject of constipation is admirably reviewed. Minor anorectal surgery and diseases of the colon, anus and rectum are discussed in the second part of the section.

Louis M. Hellman wrote the next two chapters devoted to Gynecology and Obstetrics respectively. These are notable for their brevity and lucidity. The section on sterility is especially well done. Both the normal and abnormal obstetrics are discussed as they most commonly affect the general practitioner. There is an unfortunate omission in this chapter at the bottom of page 388.

The section on Pediatrics by R. Cannon Eley is outstanding for its discussion on infant feeding and immunization procedures. The following four sections are devoted to diseases of the eye, ear, nose, throat, larynx, bronchi and esophagus. The literary styles in these vary and the first four topics are discussed with a practical point of view toward the general practitioner's management of the case. In the last section one might question the usefulness of some of the illustrations. Another highlight of the book is the most adequate chapter on dermatology and syphilology. The illustrations are good and the subject is covered with amazing brevity and fullness.

The final chapter on psychiatry contains sections on the management of neurotic patients and of patients with fatal illnesses. The essentiality of the explanatory list of psychiatric jargon could be questioned.

Although the book has been written by a number of authors, there is an absence of noticeable disjointedness of style and approach. The illustrations are profuse, appropriate and of good quality. The size and the binding of the book are such as to not make it too cumbersome. Criticisms are minor in this book which should prove of immense value to the busy general practitioner, especially to those whose practices are removed from the larger medical centers and who must of necessity perform many duties which in the larger cities would be relegated to the specialist.

J. B. MacG.

The Thyroid. By Thomas Hodge McGavack. 646 pp., $13.50. *The C. V. Mosby Co., St. Louis, Missouri.*

During recent years significant achievements have been realized in the field of endocrinology. Not the least of these attainments have been those relative to the thyroid gland. It is advantageous, therefore, to correlate these advances in order properly to adjudge and fully assess their value. Dr. McGavack, an eminent internist fully qualified to submit a text on the thyroid gland, has compiled a comprehensive and factual summary of the current concepts of thyroidology to meet the needs of the student, internist and research physician.

The Thyroid is divided into four sections. The first was written in conjunction with Dorothy B. Spear and contains an interesting historical background on thyroid disease, delineating trends which have led to present day understanding. The second section is a superlative presentation of purely anatomical, chemical and physiological considerations, giving a most thorough foundation for subsequent discussion of thyroid disease entities. Herein one may obtain authoritative data on all phases of thyroid hormone and iodine metabolism as well as the physiological interrelationships of the thyroid gland with other glands of internal secretion. Diagrams depict the relative interaction of the pituitary-thyroid axis under the influence of blocking agents. Section three includes an exhaustive classification of thyroid disease in which each disorder is fully discussed from the standpoint of pathology, histology, clinical picture, differential diagnosis and treatment. Surgical considerations are briefly outlined in section four by Drs. James W. Winfield and Walter L. Mersheimer. Surgical indications, preoperative and postoperative care and current ideals in surgical technique are discussed in practical terms.

On surveying this text one is greatly impressed by the precise organization and ease with which one may gain specific information. The data are presented concisely and logically and

are not burdensomely technical. One is also gratified by the short summaries at the conclusion of each chapter. Tables and photographs are used sparingly yet judiciously. Outstanding is the most substantial bibliography which is found as a sequel to each section. All statements throughout the text are well documented. There is a progression of thought with occasional presentations of varying points of view which allow the reader to obtain impartial data and to evaluate them as he chooses. However, the author often interprets conflicting information based on his own experience. By and large, his thoughts are those most widely accepted, and there are very few points with which one would take issue. In keeping abreast of the newer developments in the treatment of thyroid disease there is an entire chapter devoted to radioactivity and a rationale for its utilization in the treatment of disease states. This chapter will be particularly appreciated by the practicing physician since there is adequate discussion of indications and contraindications for use of radioactive iodine (I^{131}).

This refreshing and informative book places before the student and physician the basic facts correlated with recent advances and current concepts for a thorough understanding of the thyroid gland. *The Thyroid* is an authoritative publication and should be well accepted.

E. S. W., Jr.

BOOKS RECEIVED FOR REVIEW

Die Krankhafte Blutdrucksteigerung. By H. Hantschmann. 228 pp., Ganzleinen DM 36. *Georg Thieme Verlag, Stuttgart, Germany.*

Die Wuchsstofflehre. By Hans Söding. 305 pp., Ganzleinen DM 33. *Georg Thieme Verlag, Stuttgart, Germany.*

Differentialdiagnose Innerer Krankheiten. By Robert Hegglin. 456 pp., Ganzleinen DM 49.80. *Georg Thieme Verlag, Stuttgart, Germany.*

Geschwülste des Ohres und des Kleinhirnbrückenwinkels. By K. Graf. 283 pp., Ganzleinen DM 43. *Georg Thieme Verlag, Stuttgart, Germany.*

Grundlagen der Strahlentherapie. By Richard Kurt Kepp. 356 pp., Ganzleinen DM 38. *Georg Thieme Verlag, Stuttgart, Germany.*

Living in Balance. By Frank S. Caprio. 246 pp., $3.75. *The Arundel Press, Washington, D.C.*

The Merck Index of Chemicals and Drugs, 6th edition. 1167 pp., $7.50 (regular edition) $8.00 (thumb-index edition). *Merck & Co., Rahway, N. J.*

Neurosurgery. An Historical Sketch. (American Lecture Series). By Gilbert Horrax. 142 pp., $3.75. *Charles C. Thomas, Springfield, Illinois.*

Physical and Mental Stress and Consequential Development of Atherosclerosis within the Jewish Population of Denmark. By Grethe Hartmann and Fini Schulsinger. 79 pp., Dan. Kr. 8,00. *Rosenkilde and Bagger, Copenhagen, Denmark.*

Primary Carcinoma of the Liver: A Study in Incidence, Clinical Manifestations, Pathology and Aetiology. By Charles Berman. With a Foreword by Sir Ernest Kennaway and an Introduction by Henry Gluckman. 177 pp., 35s. net. *H. K. Lewis & Co., London, England.*

Problems der Schutzimpfung und Die Bekämpfung der Rindertuberkulose. By G. Ramon. 52 pp., Karton. DM 7.20. *Georg Thieme Verlag, Stuttgart, Germany.*

Progress in Allergy. (Fortschritte der Allergielehre). Vol. III. Edited by Paul Kallos. 572 pp., $16.50. *Interscience Publishers, New York.*

Psychiatrie Heute. By KURT SCHNEIDER. 32 pp., Kart. DM 2.85. *Georg Thieme Verlag, Stuttgart, Germany.*

Refraction and Motility. By WALTER B. LANCASTER. 432 pp., $7.75. *Charles C. Thomas, Springfield, Illinois.*

Signs and Symptoms, 2nd edition. Edited by CYRIL MITCHELL MACBRYDE. 783 pp., $10.00. *J. B. Lippincott Co., Philadelphia, Pa.*

Spatial Vectorcardiography. (American Monograph Series). By ARTHUR GRISHMAN and LEONARD SCHERLIS. 217 pp., $6.00. *W. B. Saunders Co., Philadelphia, Pa.*

Übungsheft für das Autogene Training. By J. H. SCHULTZ. 28 pp., Karton. DM 3. *Georg Thieme Verlag, Stuttgart, Germany.*

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